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ISOLATION OF CHOLERA VIBRIOS FROM HOOGHLY RIVER WATER AT CALCUTTA.*

BY

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OVERGROWTH by non-agglutinable ⋯⋯ ⋯ rios has hitherto been a serious inconvenience in the attempt to ⋯ ⋯ | ⋯ *choleræ* from river waters and probably accounts for certain negative findings.

The adoption of the candle-boric-peptone water method described by Panja (1942) appears to overcome this difficulty to a considerable extent. Five hundred and twenty-four samples of Hooghly river water have been examined using this technique. Three to five c.c. of unconcentrated river water from each sample were put into the porcelain candle and incubated for one or two days. A few drops from the surrounding boric-peptone water were then plated on bile-salt agar. In sixteen cases the plates subsequently showed true *Vibrio choleræ*, including both Inaba and Ogawa sub-types, the usual criteria for identification being applied. Non-agglutinable vibrios were not, however, eliminated and it is probable that if this could be done and a larger portion of water was tested. a higher proportion of positive results would be obtained.

This method was compared with attempts at the isolation of vibrios from concentrated water, using the method of filtration through filter-paper adopted

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by Dr. S. R. Pandit (unpublished) but the latter gave less satisfactory results as shown in the Table :—

TABLE.

Total number of samples.	Vibrios isolated.	Unconcentrated water.	Concentrated water.
66	Pure culture	41	23
	Mixed culture	18	34
	No vibrios	7	9

Eighty-three samples were tested on comparative lines using the candle method on the one hand and direct plating on bile-salt agar on the other hand. Fifty-two were positive for vibrios—both *V. cholerae* and non-agglutinable vibrios by the candle method—while direct plating yielded only 9 positives (non-agglutinable vibrios only).

Unfiltered Hooghly river water may, therefore, be responsible for cholera infection if used for domestic purposes. An example is known of the occurrence of cholera annually in a well-to-do orthodox family in Calcutta, who make use of Hooghly river water.

SUMMARY.

Out of 521 samples of Hooghly river water 16 showed true *Vibrio cholera* when examined by the candle-boric-peptone water method of Panja. Other methods tried were less satisfactory.

REFERENCE.

PANJA, G. (1942)   ...   *Ind. Jour. Med. Res.,* **30**, p. 391.

IMMUNITY AFTER INTRADERMAL INOCULATION OF CHOLERA VACCINE.*

BY

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DURING the war years requirements of cholera vaccine in India were greatly in excess of previous demands, either during the first world war or during epidemics in the intervening twenty years. Government laboratories were taxed to capacity and private firms were called upon to manufacture cholera vaccine on a large scale. The high cost of materials and the shortage of such items as China grass and glass-tubing increased the difficulties. The work to be described was undertaken with the object of attaining better immunization at a lower cost.

A standard cholera vaccine from the Central Research Institute, Kasauli, including both Inaba and Ogawa sub-types was used. Eleven persons were given intradermal doses of 0·1 c.c. and 0·2 c.c. at an interval of seven days, and ten persons were given the usual subcutaneous doses of 0·5 c.c. and 1 c.c. with the same interval. The persons selected for this experiment had no previous history either of an attack of cholera or of inoculation with cholera vaccine. Agglutination tests on the sera of the subjects prior to inoculation showed one with a titre of 1/10, one with a titre of 1/20 (in both cases against both Inaba and Ogawa sub-types), while the remainder showed negative results in -1 : 10 dilutions of serum. Local and general reactions after the intradermal injections were negligible.

A week after the second inoculation agglutination tests were repeated. The results are shown in Table I :—

TABLE I.

Results of agglutination tests.

Cases.	FIRST GROUP (INTRADERMAL).				Cases.	SECOND GROUP (SUBCUTANEOUS).			
	BEFORE INOCULATION.		AFTER INOCULATION.			BEFORE INOCULATION.		AFTER INOCULATION.	
	Inaba.	Ogawa.	Inaba.	Ogawa.		Inaba.	Ogawa.	Inaba.	Ogawa.
1	—	—	1/10	1/10	1	—	—	1/40	1/20
2	1/10	1/10	1/40	1/20	2	—	—	1/80	1/40
3	—	—	1/250	1/250	3	—	—	—	—
4	—	—	1/10	1/20	4	—	—	—	—
5	—	—	1/80	1/320	5	—	—	—	—
6	—	—	1/40	1/80	6	—	—	1/20	1/320
7	—	—	1/320	1/320	7	—	—	1/80	1/80
8	1/20	1/20	1/40	1/80	8	—	—	1/40	1/160
9	—	—	1/40	1/80	9	—	—	1/20	1/10
10	—	—	1/320	1/320	10	—	—	1/40	1/80
11	—	—	1/160	1/160					

— means negative 1 in 10.

All persons after intradermal inoculation showed agglutinins in titres varying from 1 in 10 to 1 in 320, thirteen out of the 22 readings being 1 in 80 or over. After subcutaneous inoculation three persons showed no cholera agglutinins (negative 1 in 10). The other seven showed titres varying from 1 in 10 to 1 in 320, only 6 out of the 20 readings being 1 in 80 or over.

The sera of the same subjects were also tested both before and after immunization for the presence of bacteriolysin and protective antibody for cholera vibrios, using Pfeiffer's test in guinea-pigs. The results are shown in Table II.

TABLE II.

Pfeiffer's test with pooled serum 0·02 c.c.

INTRADERMAL GROUP.

Pooled serum.	Pre-inoculation serum.				Post-inoculation serum.			
	Number of animals.	M	D	S	Number of animals.	M	D	S
One ...	3	+++ ++	3	0	3	—	0	3
Four ...	2	+	2	0	2	—	0	2
Two ...	2	+++ ++	2	0	2	—	0	2
One ...	2	+	2	0	2	—	0	2
TOTALS ...	9		9	0	9		0	9

SUBCUTANEOUS GROUP.

Pooled serum.	Pre-inoculation serum.				Post-inoculation serum.			
	Number of animals.	M	D	S	Number of animals.	M	D	S
One ...	3	+++	3	0	3	++	1	2
Five ...	2	+	2	0	2	+	0	2
Three ...	2	++	2	0	2	+	2	0
TOTALS ...	7		7	0	7		3	4

M = Motility of vibrios in the peritoneal fluid.   D = Animals died.   S = Animals survived.

Note.—With the pre-inoculation sera, vibrios remained motile and all test animals died.

With the post-inoculation sera after intradermal injection, vibrios were non-motile in all cases and all animals survived. With the post-inoculation sera after subcutaneous inoculation. vibrios remained motile and three out of seven test animals died. Samples of sera from the inoculated persons were not available at later periods.

Protection tests were also carried out in guinea-pigs by giving intraperitoneally 0·25 c.c. doses of pooled serum from the same inoculated persons one day before the lethal dose.

The results are shown in Table III :—

TABLE III.

Result of test for protective antibody with pooled serum 0·25 c.c.

INTRADERMAL GROUP.		SUBCUTANEOUS GROUP.	
Pre-inoculation serum.	Post-inoculation serum.	Pre-inoculation serum.	Post-inoculation serum.
3 guinea-pigs died	3 animals survived	3 animals died	3 animals survived.

N.B.—The quantity of immune serum being high, the animals in both the groups survived. Further experiment was not possible on account of shortage of serum.

With the technique and dosage used, no difference in the protective power of the sera in the two groups was demonstrable. A dose smaller than 0·25 c.c. might have shown the difference.

DISCUSSION.

The intradermal method of inoculation of cholera vaccine, therefore, appears to be superior to the subcutaneous method on the criteria of (*a*) agglutinin production in inoculated persons, (*b*) Pfeiffer's test in guinea-pigs and (*c*) protection tests in guinea-pigs. Whether these criteria are adequate when the question of mass inoculation arises is of course highly debatable. A more critical series of protective tests in animals would appear to be the next step. Advantages of the intradermal method would include economy of vaccine, only one-fifth of the usual dosage being required, and consequent economy of material and labour in production. The reaction from intradermal injection is negligible but the reaction from subcutaneous inoculation is rarely severe and not in itself a factor which has to be considered in mass inoculation. One disadvantage in intradermal inoculation would be that it would take a much longer time for mass inoculation even in experienced hands. If further work can show that one injection only would be sufficient, then it would be a great advantage.

SUMMARY.

A preliminary study on the intradermal inoculation of cholera vaccine in human subjects was carried out with encouraging results and these have received support from certain observations on experimental animals. Further work is indicated.

A METHOD OF IMPROVING THE SOLUBILITY OF DEHYDRATED CONCENTRATED HORSE SERUM IMMUNE GLOBULINS BY ADDITION OF BILE SALT AND EXTRACTION WITH ETHER.

BY

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OWING to lack of adequate and satisfactory storage facilities, the preservation of antitoxins and sera by processing them into a lyophilized form is essential in India. It has been found that a concentrated solution of horse serum immune globulins, when fractionated by the conventional method of salting out with ammonium sulphate. does not re-dissolve readily in water after being dried from the frozen state by sublimation of ice under high vacuum. Natural horse serum, however, when dried by the same process, re-dissolves rapidly. This low solubility of dehydrated concentrated immune globulins minimizes the utility of the process for the preservation of concentrated and purified antitoxins and sera. Several methods were. therefore. investigated to improve the solubility of the dried product. One of these methods has given satisfactory results and is reported here.

Low solubility of the salted out immune globulins seems to be due to the lipid containing proteins. which are separated in the same fraction. These lipoproteins are altered while drying the fraction from the frozen state (Cohn *et al.*, 1946).

The immune globulins are precipitated in fraction II+III of Cohn *et al.* (*loc. cit.*) by ethyl alcohol at controlled pH, ionic strength. temperature and protein concentration. This fraction also contains large amounts of cholesterol and other lipid substances. Therefore. the alcohol-precipitated immune globulin fraction. if not further purified by sub-fractionation. seemed likely to possess the same difficulty of preservation as the salted out immune globulin fraction. Immune globulins have. however. to be separated as a major fraction so as to facilitate and render economical large-scale processing. No economical method for the preparation of immune globulins free from lipoproteins has as yet been evolved. Pending

the availability of such an ideal method of preparing the immune globulins in a pure state, a method of improving the solubility of the major globulin fraction, dried from the frozen state, appeared to be required.

Freezing in the presence of a lipid solvent insoluble in water has been used by McFarlane (1942) as a method of removing the lipids from the proteins of the human serum to which they are attached. While about 7 g. of lipids could be removed from 1 litre of human serum by shaking with a mixture of ether and alcohol (Hardy and Gardiner, 1910), only about 4·5 g. of lipid could, however, be removed from 1 litre of the serum after exhaustive extraction by freezing in the presence of ether. A considerable amount of lipids of the globulin fraction was also found to be left behind after extraction by this method. That the residual lipids were still likely to interfere with the solubility was shown by a sample of concentrated anti-snake-venom serum, which was extracted 6 times by freezing in the presence of ether. About 3·2 g. of lipids per litre of the concentrated serum were removed. but still the dried serum did *not* show any significant improvement in solubility. Moreover, the process of repeated freezing and thawing reduced the antitoxic potency to about 80 per cent of its initial value.

Hydrophobe substances can be made hydrophile by treatment with wetting agents. Those that have been tried, being strongly polar, precipitate the proteins. The bile salts, sodium taurocholate and sodium glycocholate, on the other hand, do not precipitate the proteins but improve the solubility of the dried product. Both of the salts seemed to be equally effective. Table I shows the effect of adding different quantities of sodium taurocholate on the solubility of samples of concentrated polyvalent anti-snake-venom horse serum globulin after drying from the frozen state. The samples of the serum were dried in the Strumia type apparatus assembled by the Precision Scientific Company, Chicago. Quantities of sodium taurocholate varying between 5 mg. and 20 mg. were added to constant volumes of 10 ml. of the serum. The bile salt was intimately mixed with the serum by rolling the test-tube gently between the palms of the hands. The serum was put in ampoules of 20-ml. capacity, frozen in a slope at a temperature between 35°C. and 40°C., and then dried for 24 hours till the moisture content was reduced to less than one per cent. For determination of solubility the dried serum was dissolved in 10 ml. of distilled water at 25°C.

It was found that the bile salt must be added and intimately mixed with the serum before drying. Addition of sodium taurocholate to the dry serum did not help its solution in the least. It was also observed that if the bile salt was added 2 or 3 days before drying, the solubility of the dry serum seemed to be better than that of the sample to which the bile salt was added immediately before drying.

While analysing these results the following conclusions suggested themselves : (1) Removal of lipids by freezing in the presence of ether was not sufficient by itself to make the dried product rapidly soluble, presumably because the remaining lipids, which were still attached to the proteins, were as liable as before to be altered while drying from the frozen state. (2) Addition of bile salt to a serum, which was rich in lipids, could not improve the solubility of the dried product to the desired extent, while, when it was added to a lipid-poor serum, a very definite improvement was obtained. There seemed to exist an optimum

TABLE I.

Solubility of dehydrated concentrated anti-snake-venom horse serum globulin containing different amounts of sodium taurocholate.

Batch number of serum.	Amount of sodium tauro-cholate added, mg. per ml.	Time required by the dry serum to dissolve completely in water, minutes.	Percentage of dry serum dissolving in one minute.*
	Nil	55†	32†
	0·5	14	80
22	1·0	11	86
	1·5	12	88
	2·0	11	82
	Nil	70	19
	0·5	16	68
30	1·0	14	72
	1·5	12	77
	2·0	12	70
	Nil	180	15
	0·5	75	54
37	1·0	60	62
	1·5	60	51
	2·0	45	60

* The percentage of dry serum dissolving in one minute was determined in the following way: The total protein content of 10 ml. of the liquid serum was determined by Kjeldahl's method before drying. After addition of 10 ml. of distilled water to the dry serum, the ampoule was shaken vigorously for one minute: A small amount of octyl alcohol was added with a platinum loop to dissipate the froth formed by shaking. As soon as the froth disappeared, the whole of the dissolved serum was pipetted off to a calibrated measuring cylinder, its volume was measured, and the protein content of the total volume was determined. The ratio of the total amount of protein in the volume of the serum recovered in solution, to that of 10 ml. of the original liquid serum, multiplied by 100, gave the percentage of dry serum dissolving in one minute.

† The figures are the approximate arithmetic means of determinations on 3 different samples.

TABLE I—*concld.*

Batch number of serum.	Amount of sodium tauro-cholate added, mg. per ml.	Time required by the dry serum to dissolve completely in water, minutes.	Percentage of dry serum dissolving in one minute.*
	Nil	150	17
	0·5	17	66
37† lipid poor	1·0	14	71
	1·5	15	69
	2·0	15	73

* The percentage of dry serum dissolving in one minute was determined in the following way: The total protein content of 10 ml. of the liquid serum was determined by Kjeldahl's method before drying. After addition of 10 ml. of distilled water to the dry serum, the ampoule was shaken vigorously for one minute. A small amount of octyl alcohol was added with a platinum loop to dissipate the froth formed by shaking. As soon as the froth disappeared, the whole of the dissolved serum was pipetted off to a calibrated measuring cylinder, its volume was measured, and the protein content of the total volume was determined. The ratio of the total amount of protein in the volume of the serum recovered in solution. to that of 10 ml. of the original liquid serum, multiplied by 100, gave the percentage of dry serum dissolving in one minute.

† Serum batch 37, which was extracted 6 times by freezing in the presence of ether, and from which 3·2 g. of lipids were removed per litre of the serum.

for the useful concentration of the bile salt, which was ordinarily 1 mg. per ml. Being limited by this optimum, the bile salt could act only on a limited amount of lipoproteins. Any excess lipoproteins remaining after the addition of the optimum concentration of the bile salt was unstable and was altered while drying from the frozen state. The reason for the existence of this optimum for the active concentration of the bile salt was, however, not understood. (3) Much less lipids (0·5 g. per litre) were separated from the serum (batch 22) by natural forces, while maturing in storage for about 5 months at 0°C. compared with the amounts (3·2 g. per litre) that were removed from the serum (batch 37) by exhaustive extraction with ether. Yet, the former dissolved much more rapidly than the latter when both were dried under the same conditions. It appeared that during maturation. not only did some of the lipids separate out from the proteins. but some of the others which still remained attached to the proteins, had their linkages firmly fixed, so that they did not alter while drying from frozen state.

It was clear that. in order to gain the maximum advantage out of the bile salt, a part of the lipids in the horse serum globulin fraction required to be removed.

It has been mentioned before that the method of extraction of these lipids by freezing in the presence of ether was not found suitable, chiefly because the anti-toxic potency was reduced during extraction. Alternative methods of extraction had, therefore, to be investigated, and the following method was evolved :--

Ether could extract a considerable amount of lipids provided a small quantity of bile salt was previously intimately mixed with the globulin fraction. After the lipids thus extracted with the bile salt and ether were separated out of the fraction, further extraction with ether alone was fruitless. As soon as some more bile salt was added to the fraction, ether again succeeded in removing further quantities of lipids. This process of extraction required to be repeated a few times. in certain cases, before the globulins, after drying, dissolved sufficiently rapidly in water. The extraction could be carried out a number of times even after the desired solubility was obtained, but no further advantage was gained. On the contrary, the solution became opalescent owing to emulsion of ether in serum and it became increasingly difficult to filter it through sterilizing asbestos-paper-pulp pads. The amount of lipids extracted from the fraction with ether, in the presence of bile salt varied from batch to batch. The protocol of the method of large-scale extraction of lipids is given below.

The concentrated anti-snake-venom serum. which was fractionated in small lots, was pooled together, its pH adjusted to 7·2 and sodium-chloride was added to make a content of 0·85 per cent. The serum, after clarification through clari-fying asbestos-paper-pulp pads, was distributed in 6-litre amounts into 9-litre Pyrex glass serum bottles. The serum was cooled to 0°C. and then 6 g. of sodium tauro-cholate was added to each bottle. The bile salt was intimately mixed with the serum by prolonged gentle agitation short of formation of any froth. After thorough mixing. the serum was stored at 0°C. for 2 days. For extraction of the lipids. 2 litres of ether, cooled to 0°C., were added to each bottle. The serum and the ether were intimately mixed by vigorous shaking. The mixture was decanted into one-litre Pyrex centrifuge bottles, which were filled up to capacity, and their mouths closed with rubber-caps. After centrifuging for 15 minutes at 2,000 r.p.m. at room temperature (28°C. to 32°C.). 3 layers were formed in each bottle. The top consist-ed of excess of ether, the intermediate of extracted condensed lipids of white colour, and the bottom layer of clear serum, which was siphoned off. The siphoned serum was pooled in 9-litre bottles and the dissolved ether was removed by blowing compressed air through a jet over the surface of the serum at 25°C. After ether had been removed, 1 g. of sodium taurocholate was added per litre of the serum and intimately mixed. Two days later, samples of 10 ml. of the serum were dried from the frozen state, and the solubility of the dried product tested. If the solubility was not found to be satisfactory, the serum was extracted with ether in the presence of 0·1 per cent sodium taurocholate as before.

None of the serum. so far dried, required extraction for more than 4 times. A sample of serum which was extracted 4 times was usually sufficiently soluble without the addition of more bile salt. The serum, which required fewer extractions, generally required 0·1 per cent bile salt before showing satisfactory solubility. Hence, while no bile salt was added to the serum after the 4th extraction, 0·1 per cent of it was always added to those that were extracted less than 4 times.

For the determination of the amount of lipids extracted, the fatty layers in 8 centrifuge bottles were collected together in a large porcelain dish, and both ether and moisture were removed by heating over a water-bath at 95°C. to constant weight. The weight of the residue, divided by 6, gave the amount of lipids extracted per litre of the serum. This amount differed in different batches. The rate of extraction from a serum at different steps was also irregular. For instance, out of each litre of the serum (batch 37), 0·8 g. was removed during the first extraction, 1·2 g. during the second, 0·4 g. during the third, and 0·3 g. during the fourth. Table II shows the solubility of 3 different batches of concentrated anti-snake-venom serum extracted with ether in the presence of the bile salt :—

TABLE II.

Solubility of dehydrated concentrated anti-snake-venom horse serum globulin containing 0·1 per cent bile salt and that of the same serum extracted with ether in the presence of bile salt.

Batch number of serum.	Method of treatment.*	Amount of lipids extracted per litre, g.	Time required by the dry serum to dissolve completely in water, minutes.‡	Percentage of dry serum dissolving in one minute.†
22	A	0	10†	85†
	B	1·2	4	100
30	A	0	15	70
	B	2·0	3½	100
37	A	0	55	60
	B	2·7	5	100

* Treatment A consisted of adding 1 mg. of sodium taurocholate per ml. of the serum. Treatment B consisted of extracting the serum with ether after addition of the bile salt. Extraction was repeated twice in the case of batch 22 and 4 times each in the cases of batches 30 and 37.

† *See footnote marked * of Table I for clarification.*

‡ Over 99 per cent of the dried product went into solution during the stated period. A trace of globulins, which formed complexes with carbohydrates, however, required a much longer time to dissolve.

The results show that extraction of lipids with ether in the presence of the bile salt improves the solubility of the dry product out of all proportion to the small amount of lipids that are removed. A reference to the solubility figures, corresponding to the serum batch 37 in Table I and Table II, will show the difference. While 3·2 g. of lipids were removed from the serum batch 37 (Table I) by freezing in the presence of ether, only 2·7 g. were removed from the same by ether in the presence of the bile salt (Table II). Yet the latter, when dry, dissolved much more rapidly than the former. Evidently, the active concentration optimum,

which was initially 1 mg. of bile salt per ml. of the serum, had been shifted on a higher level during these extractions. Prior to this shift of the optimum, a considerable amount of lipoproteins could not come within the sphere of activity, which was limited by the existing active concentration optimum, of the bile salt. After the shift, however, more and more of the bile salt became active, and its sphere of activity was extended. The bulk of the lipoproteins could, therefore, now be influenced by the bile salt with a considerably extended sphere of activity. Thus influenced by the bile salt, the lipoproteins no longer became altered while drying from the frozen state. Hence, the dried product dissolved rapidly in water. The mode of action of the bile salt upon the lipoproteins was not, however, clearly understood.

It was remarkable that the serum, which had been so violently treated during repeated extractions with ether after addition of bile salt did not show any less of antitoxic potency. Nor was it any more unsafe for use in man in spite of the presence of considerable amounts of the bile salt. On the contrary, the extracted serum was better than the original serum, in that it was less viscous than before and presented a crystal-clear appearance.

SUMMARY.

Concentrated horse serum globulin fraction, which contained the anti-snake-venom immune bodies, re-dissolved, when dried from the frozen state, very slowly in water. If, however, 0·1 per cent of sodium taurocholate was added to the globulin fraction, and the fraction was then extracted with excess of ether, a small amount of lipids was removed from the fraction, and the fraction dissolved rapidly in water after drying from the frozen state. Neither the extraction of lipids alone, nor the addition of the bile salt alone, was enough to improve the solubility of the dried product to the desired extent. Extraction of lipids with ether in the presence of the bile salt improved solubility without any loss of the immune bodies and without making the product unsafe for use in man.

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HYPOPROTHROMBINÆMIA PRODUCED BY SULPHATHIA-ZOLE IN RATS ON A DIET FREE OF VITAMIN K AND CURED BY SYNTHETIC VITAMIN K.

BY

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DAM, Schonheyder and Tage-Hansen (1936) were the first to demonstrate that the absence of vitamin K produced in the chick a hæmorrhagic syndrome character-ized by a prolongation of prothrombin time. Since then many attempts have been made to produce the same symptoms in common laboratory animals like the rat, rabbit or guinea-pig. Thus Elliot, Isaacs and Ivy (1940) produced a deficiency of prothrombin in the rat by sustaining them on a diet containing 20 per cent mineral oil by weight. Recently, dicoumarol (Witts, 1942 ; Overman, Stahmann, Sullivan, Huebner, Campbell and Link, 1942) has also been utilized to produce hypopro-thrombinæmia which can be cured by the administration of vitamin K. Most other procedures previously used to produce vitamin K deficiency in the common laboratory animals make use of some form of surgical procedure which inhibits the absorption of vitamin K, thereby causing a deficiency of this vitamin in the animal.

The work of Greaves and Schmidt (1937) as well as that of Dam, Schonheyder and Lewis (1937) shows that mammals in general are not very susceptible to a purely dietary vitamin K deficiency.

The observations that *coliform* organisms produce vitamin K *in vitro* (Greaves, 1939) and that fæces of rats on a diet free from vitamin K contain vitamin K (Orla-Jensen, Orla-Jensen, Dam and Glavind, 1941) have recently led to the development

of methods for producing a deficiency of this vitamin in the rat, by the inhibition of intestinal synthesis of vitamin K with sulphonamide drugs.

Daft, Ashburn and Sebrell (1942), Kornberg, Daft and Sebrell (1944a, 1944b), as well as Black, Overman, Elvehjem and Link (1942), report that continual feeding of sulphonamide drugs to rats together with a synthetic ration free from vitamin K can consistently produce hypoprothrombinæmia.

These findings suggest a convenient method for producing vitamin K deficiency in the rat, and possibly the development of a curative method for the assay of this vitamin.

With this object, further data are presented concerning the production of hypoprothrombinæmia in the rat using sulphathiazole in conjunction with a diet free from vitamin K, and its correction with synthetic vitamin K. A preliminary note on the subject was published elsewhere (Braganca and Rao, 1946).

EXPERIMENTAL PROCEDURE.

Three groups of male albino rats from the Haffkine Institute colony, about 25 days old, and weighing 25 to 40 grammes were used.

The synthetic ration given to the animals was the same as that used by Kornberg *et al.* (1944a) with the exception that the vitamin B-complex supplement was supplied in the form of dried brewer's yeast, previously extracted with ether in order to remove any vitamin K present.

The first group consisted of six animals kept on the vitamin K-free ration supplemented with sulphathiazole at 1 per cent level and 100 μg. of synthetic vitamin K ['Synkavit' (Roche)] by mouth per week.

The second group which consisted of 20 animals received the same synthetic diet as the first, but without the vitamin K supplement.

The third group consisted of six animals and received the same synthetic ration as the above groups, but without the supplement of vitamin K or the drug.

Along with the groups of rats mentioned above, 5 rats in addition were kept on the same ration as that of group 2. At the end of three weeks when they had developed severe hypoprothrombinæmia, they were killed and the condition of liver studied histologically. Besides these, most of the animals that died, as well as some selected animals from the different groups, were killed at definite periods and the livers examined histologically. The results are summarized at the end of the paper.

Few animals were used in groups one and three as preliminary experiments had shown that most of the animals receiving similar diets survived for long periods.

Prothrombin estimations were made by a modification of the micro-method of Innes and Davidson (1941) in which the prothrombin in the first drop of blood obtained by clipping the tail was determined. As it is well established that the normal prothrombin time for each species is fairly constant, the prothrombin time of the tail blood of normal rats of similar weight as those used in the experiments was previously established by using the same technique. The values obtained ranged from 15 to 20 seconds.

The thromboplastin used in the prothrombin estimations was Russell's viper venom in 1 : 10,000 dilution. The crude venom extracted in the Haffkine Institute (representing a sample of pooled venom from several Russell's vipers) was purified by re-dissolving in water, centrifuging at high speed to remove the mucus, and drying it by the lyophile process. The stock solution of 1 : 1,000 dilution could be kept in the cold at 4°C. without any change in potency for one month.

The supplement of vitamin K in the form of ' Synkavit ' (Roche) was given orally once a week, diluted in such a way that 0·1 c.c. contained the required dose.

RESULTS.

It was found that for the first two weeks the animals of all the groups looked quite healthy and took food liberally. During the third week most of the animals of the second group showed an abrupt change in their general condition. They appeared weak and extremely pale (as seen from the eyes and ears), while some showed bleeding from the ears, nose and mouth. Two animals in this group died in 17 and 20 days respectively before the prothrombin time was determined and in the others the determinations were made during the third week.

Large single doses of vitamin K (100 μg.) in the form of ' Synkavit ' (Roche) were then administered orally to the animals showing a prolonged prothrombin time (30 seconds or more) and the reaction of animals to this dose noted by another prothrombin determination carried out after forty-eight hours. The results are given in Table I. Fifteen out of 18 animals showed a prolongation of prothrombin time (Nos. 27 and 32 being killed after two weeks to find whether there was any abnormality in the internal organs), of which ten reacted to the dose of vitamin K given. Considering the animals that reacted successfully to doses of vitamin K, it is seen that Nos. 25 and 26 were given 50 μg. vitamin K whilst the rest received 100 μg. Though both doses were sufficient to correct the hypoprothrombinæmic state, it is noteworthy that the prothrombin time of the majority of animals receiving the larger dose returned to a value less than the minimum prothrombin time obtained for normal rats (15 seconds).

The records of rats Nos. 14, 17 and 30 are particularly interesting. They show the gradual fall in prothrombin time as their stores of vitamin K are depleted and their successful reaction to repeated vitamin K administration. In the case of rat No. 30 it is further seen that at a later stage 100 μg. of vitamin K could maintain the normal prothrombin level for a period of 79 days. In this connection it may be mentioned that in another experiment in which bigger animals were used (45 to 60 grammes) difficulties were experienced in trying to produce hypoprothrombinæmia by the same method within a month. It was found that the animals did not show a fall of prothrombin for a period of 6 weeks. These observations suggest a possible relationship between the age of the animal and its susceptibility to hypoprothrombinæmia produced by sulphathiazole, and emphasizes the importance of age and weight of the animal in such experiments.

In contrast to the behaviour of animals to the second group, it will be seen from Tables II and III that those belonging to the first and third groups remained healthy and showed a normal prothrombin time for a period of over two months

TABLE I.

(Group 2.)

Animals of this group received vitamin K-free diet containing sulphathiazole.

Number of rat.	Number of days on diet.	Prothrombin time in seconds.	Dose of vitamin K given (μg.).	Number of days since giving vitamin K.	PROTHROMBIN TIME IN SECONDS AFTER RECEIVING VITAMIN K, PERIOD BETWEEN TWO CONSECUTIVE DETERMINATIONS AND DOSE OF VITAMIN K GIVEN.							
					(1)		(2)			(3)		(4)
					Prothrombin time.	Number of days.	Prothrombin time.	Dose of vitamin K (μg.).	Number of days.	Prothrombin time.	Number of days.	Prothrombin time.
14	18	45	100	2	12	7	25	...	5	80	...	...
17	21	80	100	2	12	3	20	...	10	120	...	...
21	21	100	100	2	13	...	...	...	...	...	...	...
23	20	70	100	2	10	...	...	...	...	...	...	...
25	18	75	50	2	17	...	...	...	...	...	...	...
26	18	70	50	2	18	...	...	...	...	...	...	...
28	18	90	100	2	15	...	...	...	...	...	...	...
29	18	80	100	2	15	...	...	...	...	...	...	...
30	20	75	100	2	8	14	100	100	39	20	40	100
31	20	70	100	2	12	...	...	...	...	...	...	...
13	17	Died	...	...	...	...	...	...	...	...	...	...
15	17	115	100	Died	...	...	...	...	...	...	...	...
16	17	80	100	,,	...	...	...	...	...	...	...	...
18	21	25	...	,,	...	...	...	...	...	...	...	...
19	21	80	100	,,	...	...	...	...	...	...	...	...
20	20	Died	...	...	...	...	...	...	...	...	...	...
22	18	75	100	Died	...	...	...	...	...	...	...	...
24	22	80	100	,,	...	...	...	...	...	...	...	...
27	12	15	...	Killed	...	...	...	...	...	...	...	...
32	12	30	...	,,	...	...	...	...	...	...	...	...

TABLE II.

(Group 1.)

Animals of this group received vitamin K-free diet containing 1 per cent sulphathiazole and 100 µg. vitamin K per week.

Number of rat.	Number of days on diet.	Prothrombin time in seconds.	Number of days on diet.	Prothrombin time in seconds.	Number of days on diet.	Prothrombin time in seconds.
7	16	17	44	15	60	15
8	20	17	Killed	...	...	...
9	24	16	44	17	72	18
10	19	18	44	15	62	18
11	Died	...	...	...	...	...
12	19	17	38	20	67	18

TABLE III.

(Group 3.)

Animals of this group received vitamin K-free diet.

Number of rat.	Number of days on diet.	Prothrombin time in seconds.	Number of days on diet.	Prothrombin time in seconds.	Number of days on diet.	Prothrombin time in seconds.
1	24	18	47	18	60	17
2	24	17	47	18	60	15
3	24	16	47	15	60	17
4	24	16	47	18	60	18
5	24	16	47	18	60	18
6	24	17	47	18	60	18

when most of them were killed. One animal (No. 6) belonging to the group receiving vitamin K-free ration was, however, continued on this diet for over 4 months. On the 127th day it was found to be bleeding from the mouth and nose and examination showed a prothrombin time of 100 seconds. Although 100 µg. of vitamin K was administered the animal did not survive. These observations confirm the findings of other workers that rats are not very susceptible to a pure dietary deficiency of vitamin K.

PATHOLOGICAL FINDINGS.

In order to study the effect of continued feeding of sulphathiazole together with a vitamin K-free diet on the liver and other organs, some animals were killed when severe hypoprothrombinæmia had developed. It was found that in the majority of cases the liver was enlarged and pale, as also the kidneys. Hæmorrhages were found in various parts of the body, the most common sites being the subcutaneous tissues, the urinary bladder, the epididymis, testicles and the pelvic region.

Histological examination of the liver and kidneys showed the following changes :—

In the case of animals receiving the drug together with a vitamin K-free diet, it was found that till the end of the second week there was practically no change in the histology of the liver. During the third week, however, marked prolongation of prothrombin time was generally associated with considerable fatty change in the liver. Necrosis of the parenchyma around the central veins was evident in some cases. Animals of this group were not kept for more than 24 days.

In a few cases the kidneys were also examined and stained sections showed slight cloudy swelling of the epithelium of the convoluted tubules.

The livers of animals in the group receiving vitamin K and the drug showed practically normal histological appearances, even though these animals had been receiving the drug for periods varying from 60 to 72 days. The kidneys, however, showed cloudy swelling of the tubular epithelium similar to that seen in the above group, which received vitamin K-free diet and the drug.

It is thus evident that the addition of vitamin K to a synthetic vitamin K-free diet to which sulphathiazole has been added, prevents the fatty change in the liver, as determined histologically. On the other hand, histological changes in the kidneys could not be prevented by the addition of vitamin K.

The possibility of developing the above procedure for producing vitamin K deficiency in the rat into a curative method for the assay of vitamin K is now being investigated. Preliminary experiments, planned with a view to determine the minimum dose of vitamin K which will cure the hypoprothrombinæmia produced by sulphathiazole, have indicated that the dose is about 5 μg. The data are not reported here as it is felt that the results require confirmation with a larger group of animals.

SUMMARY.

1. Severe hypoprothrombinæmia was produced in the white rat by feeding for a period of three weeks a diet free from vitamin K and containing 1 per cent sulphathiazole. This condition was prevented by the addition of vitamin K to the diet.

2. The hypoprothrombinæmia so produced was cured by the oral administration of a single dose of 50 to 100 micrograms of synthetic vitamin K. The prothrombin time of the animals which received 100 micrograms of vitamin K returned to a value, which was less than the minimum obtained for normal animals, in about 48 hours.

3. Older animals were found to be less susceptible to the production of vitamin K deficiency by feeding sulphathiazole.

4. A deficiency of vitamin K by purely dietary means was produced only in rare instances.

5. Histopathological investigations showed that continued feeding of the drug along with a diet free from vitamin K produced considerable fatty change in the liver and the condition was prevented by the addition of synthetic vitamin K to the diet.

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THE BIOLOGICAL VALUE OF THE PROTEINS OF RICE, PULSE AND MILK FED IN DIFFERENT PROPORTIONS TO HUMAN BEINGS.

BY

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AND

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(*An Inquiry under the Indian Research Fund Association.*)

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INTRODUCTION.

DIETARY surveys carried out all over India show that protein from cereals and pulses forms the major source of nitrogen in the diet of the people of this country. Basu and Basak (1939) were pioneers in determining the biological value of mixed proteins in a diet consisting of rice, pulse and vegetables by human feeding experiments. In their series of experiments, which were conducted on two adult male subjects, the pulse quota (25 g.) in the test diets was constant and the amount of cereal included was 600 g. In the present investigation an attempt has been made to find out whether the rice-protein and pulse-protein ratio influences the total biological value : whether, in other words, the inclusion of a greater quantity of pulses in a rice diet improves its biological value in respect of protein. The ratios of proteins from pulses and rice respectively were fixed at 75 : 100, 100 : 100 and 125 : 100 in the three different test diets given to the experimental subjects. Finally, in order to find out whether the substitution of pulse proteins by milk proteins made any difference in nitrogen balance, in the subsequent series of experiments, the pulse proteins were completely replaced by equivalent amounts of milk protein at all the three levels of feeding.

(23)

EXPERIMENTAL.

Six healthy human subjects, M. R. (21 years) weighing 41 kg., M. M. (24 years) weighing 40 kg., S. A. (39 years) weighing 52·5 kg., I. L. (30 years) weighing 48 kg., B. S. (28 years) and B. P. (26 years) each weighing 47 kg., were kept on each of the test diets for a period of one week. Before the test diets were given each of the subjects was kept on ' low nitrogen ' diet for a period of one week to determine endogenous nitrogen metabolism for each subject. In preference to a ' non-nitrogenous ' diet a ' low nitrogen ' diet was given, as it was felt that a diet consisting of sugar, sago and starch, besides being monotonous and unpalatable, may not evoke the usual gastro-intestinal response in the subjects and the digestive organs might not function properly with an unusual kind of prepared food. The psychological factor may be ignored with rats and dogs as experimental subjects in feeding experiments, but with human subjects it was felt that data collected after the use of unaccustomed type of diets might lead to erroneous results. During the ' low nitrogen ' period, breakfast consisted of a popular Indian concoction, ' halwa ', made of sugar, sago and ghee or hydrogenated fat. The noonday meal consisted of halwa, mashed potato, gourd and leafy vegetables cooked in mustard oil. The evening meal consisted of biscuits made of nitrogen-free corn starch and prepared by a local firm of caterers. An attempt was made to bake loaves out of starch on the lines suggested by Summer and Murlin (1938), but even after a week's trial, baking was not successful. Consequently biscuits were made by using the following ingredients in the proportions indicated :—

Starch	...	100 g.
Ghee and hydrogenated fat	...	300 g.
Sugar	...	250 g.
Honey	...	180 g.
Baking powder and table salt	...	qs.

Efforts to prepare ' chapattis ' (unleavened bread) by the addition of mucilage to the starch as binding material failed. The biscuits were eaten with honey, mashed potato, gourd and leafy vegetables cooked in fat. No condiments were used in cooking the vegetable servings, but enough table salt was used to make the food palatable. Juice from one ordinary sized bazaar lemon was served daily to each subject along with the food. All the subjects were found to relish the meals. Each subject received 3 tablets of Squibb's ' Vita-Yeast ' tablets daily. The amount of nitrogen obtained from the vegetables rarely exceeded 9 per cent of the total intake.

Samples of stool and urine were collected daily, but only the last three days' collection for each seven-day period has been taken into account. Urine for 24 hours was collected in enamel chamber-pots containing 10 c.c. of a 10 per cent solution of thymol and 20 c.c. of a 5 per cent solution of phenol. The fæces were collected in separate pots containing acetic acid and each collection for a period of 24 hours was dried on a water-bath with frequent additions of rectified spirit and acetic acid. The dried fæces for the last three-day period were pooled together, ground to fine powder, weighed and aliquots taken for estimation of nitrogen in the respective samples. The urine after each twenty-four-hour collection was

measured and subjected to analysis for estimation of nitrogen by the Kjeldahl method. Aliquot portions of the samples of food were also collected daily, dried in a water-oven and the amount of nitrogen therein estimated.

TABLE I.

Data for the low nitrogen diet.

Name of subjects.	Weight of the subject, kg.	Total dry weight of food, g.	Weight of nitrogen in the food, g.	OUTPUT OF NITROGEN.		
				Urine, g.	Fæces, g.	TOTAL, g.
M. R. ...	41·0	820	0·31	1·414	0·710	2·124
M. M. ...	40·0	820	0·31	1·405	0·746	2·151
S. A. ...	52·5	990	0·50	1·899	1·220	3·119
I. L. ...	47	990	0·50	1·487	1·170	2·657
R. S. ...	47	830	0·54	1·587	0·958	2·545
B. P. ...	47	830	0·54	1·785	1·095	2·870

All these subjects were put on each of the three test diets, consisting of rice and pulses, for three consecutive weeks, and then after an interval of 15 days the 3 rice and milk periods of one week each followed. During the rice and pulse period the proportion of rice protein : pulse protein was 100 : 75 throughout the first week, 100 : 100 in the second week and 100 : 125 in the final week. During the rice and milk period the proportions of rice protein : milk protein were 100 : 75, 100 : 100, and 100 : 125 in the first, second and third week respectively as in the case of pulse period. The details of the test diets are given in Table II. Two subjects, B. S. and B. P., were ordinarily accustomed to an afternoon snack and they were supplied with halwa. The fat used for cooking was either ghee or hydrogenated groundnut oil. Just enough for cooking purposes was used and the amount varied from 35 g. to 45 g. Rice was boiled and gruel was made of pulses : they were not cooked together or made into 'khichri'. Similarly, in the milk period whole milk was boiled in a pan and a mixture of boiled rice and milk was taken with a little quantity of added sugar. No vitamin supplements were allowed. One of the subjects (S. A.) was accustomed to take tea but he willingly refrained from the beverage during the experimental periods.

TABLE II.

*Composition of test diets.**

Name of subjects.	RICE PROTEIN: PULSE PROTEIN. 100 : 75		RICE PROTEIN: PULSE PROTEIN. 100 : 100		RICE PROTEIN: PULSE PROTEIN. 100 : 125	
	Rice, g.	Pulse, g.	Rice, g.	Pulse, g.	Rice, g.	Pulse, g.
M. R. ...	550	114	480	136	427	151
M. M. ...	550	114	480	136	427	151
S. A. ...	634	135	556	157	494	175
I. L. ...	634	135	556	157	494	175
B. S. ...	460	98	402	115	358	125
B. P. ...	460	98	402	115	358	125

Pulses completely replaced by milk.

Name of subjects.	Rice, g.	Milk, ml.	Rice, g.	Milk, ml.	Rice, g.	Milk, ml.
M. R. ...	550	838	480	971	427	1,082
M. M. ...	550	838	480	971	427	1,082
S. A. ...	709	1,080	620	1,265	544	1.465
I. L. ...	709	1,080	620	1,265	544	1,465
B. S. ...	460	702	402	815	358	908
B. P. ...	460	702	402	815	358	908

* In addition to above the diets consisted of :—

Cooking fat	...	...	...	35 g. to 45 g.
Potato ...	...	...	...	120 g.
Bottle gourd or vegetable marrow		...	150 g.
Leafy vegetable	...	...	...	150 g.
In the milk group, sugar		...	...	30 g.

RESULTS.

The mean biological value was calculated according to the following formula :—

$$B.V. = 100 \times \frac{\text{Body nitrogen retained}}{\text{Food nitrogen absorbed}}$$

or in other words

$$100 \times 1 - \frac{\text{Total urine N} - \text{endogenous urine N}}{\text{Food N} - (\text{fæcal N} - \text{metabolic N})}$$

The biological values are shown in Table III :—

TABLE III.

Biological values.

Name of subjects.	Rice protein : Pulse protein.			Rice protein : Milk protein.		
	100 : 75	100 : 100	100 : 125	100 : 75	100 : 100	100 : 125
M. R. ...	56	59	59	66	63	68
M. M. ...	61	54	51	67	60	65
S. A. ...	54	56	61	66	69	64
I. L. ...	54	61	58	71	68	63
B. S. ...	64	58	62	68	70	67
B. P. ...	57	64	54	69	63	68
Mean biological value.	57·8	59·2	56·4	67·8	66·5	65·5

The mean biological values come to 57·8, 59·2 and 56·4 in the case of ratios of rice protein to pulse protein at 100 : 75, 100 : 100 and 100 : 125 feeding levels respectively. The respective figures for milk and rice protein were 67·8, 66·5 and 65·5. The average biological value of the proteins of a rice diet (with only 25 g. of pulses) was found to be 75 by Basu and Basak (*loc. cit.*) in Dacca, whereas in the present investigation it was approximately 58 with the pulse quota in the diet varying from 98 g. to 175 g. in the different test diets. The increase in the pulse quota may have been responsible for the lowering of biological value. With different quantities of milk also one would have expected a slightly higher biological value, but it may be noted that the protein level in the test diets was in the neighbourhood of 12 per cent instead of the 5 per cent level allowed to the subjects at Dacca. The figures were subjected to statistical analysis and it was found that the differences noticed within the group with the same type of diet, i.e. rice and pulse proteins or rice and milk proteins, were not significant. In other words, the proportion of rice protein and pulse protein in the diet at the

levels under investigation made little or no difference in the biological value. The milk protein also behaved in the same way. The difference between the groups, i.e. milk and pulses, was, however, statistically significant and the former was found to possess a definitely superior biological value.

SUMMARY.

The biological value of the proteins of mixtures of rice and pulse fed in different proportions to six human volunteers varied between 54 and 64. The corresponding values, when milk was substituted for pulses, varied between 60 and 71.

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THE FOOD HABITS OF THE MUSLIMS OF BIHAR AND THE NUTRITIONAL STATE OF THEIR CHILDREN.

BY

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INTRODUCTION.

AN attempt has been made to study the food habits of the Muslim community in Bihar living in rural areas and belonging to different economic, social and occupational groups and also the nutritional state of their children. The present inquiry was undertaken as no records of a large-scale dietary and nutrition survey solely confined to Muslims are available. Investigations on families residing in urban areas were not undertaken for various reasons, the principal one being that life in cities often necessitates and results in a change of traditional dietary habits. The localities selected for survey in rural areas were such that the villages and their neighbourhood contained predominantly a Muslim population, and the families in each group studied consisted of the people belonging more or less to the same economic and social groups. Families with monthly incomes approaching Rs. 500 or above have been excluded, as it was considered that their inclusion might interfere seriously with the average figures in the higher income groups. The families were sampled from seven civil districts of the province. The examination of children, however, could not be confined to the families in which dietary investigations were carried out, since if this had been done the numbers examined in each of the nine localities surveyed would necessarily have been too small for the purposes of study. Consequently, in each of the centres of investigation almost all children between the ages 2 and 14 years residing in and around the villages where dietary surveys were carried out were examined clinically and anthropometrically.

DIETARY INVESTIGATIONS.

Dietary surveys, covering a period of 10 days in each family, were started early in the year 1939 and continued till the month of February 1943 on the lines detailed in a previous communication from these Laboratories (Mitra, 1940). The

investigations were completed before the unusual rise in the prices of food-grains started in 1943. In the nine localities the food intake of 709 Muslim families, including a total population of 4,789 persons, was investigated. In the districts of Saran and Patna the families surveyed were economically better off, being tenant cultivators and cultivating proprietors (upper-middle class).

<div align="center">TABLE I.</div>

The distribution of families in the different districts and their average income and composition.

Group symbols.	District.	Special sub-groups.	Number of families surveyed.	Average number of consumers per family.	Average number of consumption units (c.u.).	Average income per c.u. per diem in annas.
A ...	Saran	Upper-middle class.	80	9·0	6·9	9·2
B ...	Patna	,,	120	7·3	5·6	8·0
C ...	Gaya	Momins	20	7·3	5·3	7·5
D ...	Gaya	Lower-middle class.	143	6·8	4·4	5·2
E ...	Shahabad	,,	55	5·8	4·4	5·2
F ...	Purnea	Jute culti- vators.	50	5·0	3·7	4·4
G ...	Purnea	Sisbadhia (cultivators).	60	8·7	6·4	3·7
H ...	Darbhanga	Poor Pathans (cultivators).	100	6·7	4·9	2·8
·K ...	Santhal Perganas.	Poor Momins (weavers and cultivators).	81	4·9	3·8	1·7

In Gaya and Shahabad districts (D and E) the families were tenant cultivators but of the lower-middle class. The Momins are mostly weavers by tradition but the families surveyed in Gaya district (C) were tenant cultivators, whereas those belonging to the district of Santhal Perganas (K) were weavers and agricultural labourers, though most of them had very small agricultural holdings of their own. The weavers also were to some extent economically distressed during the period of survey as the price of cotton yarn had increased with no proportionate increase in the price of the coarse cloth which they produced. The jute cultivator group of families (F) were not so well off as previously, since during the year of survey (1942). jute, the main cash crop, was not fetching the usual good prices. Another point which deserves mention is that people in this particular locality are victims of hyperendemic malaria and they have to employ labour from outside on higher

wages as compared to wages prevalent in other parts of this province. The Sisbadhia Muslims (G) claim to have migrated from other parts of India. They live by cultivation (and at times by fishing) in the fertile Gangetic valley. Both the Sisbadhia (G) and jute cultivator group (F) of families in the district of Purnea were mostly composed of tenant cultivators ; the families surveyed in Darbhanga district were poorer-class tenant cultivators and also agricultural labourers.

TABLE II.

Consumption of different classes of food per c.u. per diem in oz.

Serial symbol.	Cereals.	Pulses.	Leafy vegetables.	Non-leafy vegetables.	Fats and oils.	Flesh foods.	Milk and milk products.	Fruits and nuts.	Condiments.	Other foods.
A ...	22·3	4·8	0·3	4·6	1·3	3·7	5·2	0·3	1·4	0·9
B ...	18·0	4·1	1·2	5·9	1·8	3·7	4·1	0·6	1·8	0·6
C ...	23·2	6·2	0·7	4·7	0·9	1·5	4·3	0·1	1·2	0·8
D ...	20·0	6·0	0·7	3·5	0·8	1·7	3·3	0·2	1·1	0·4
E ...	18·6	2·8	0·5	2·5	0·9	2·2	1·1	*Nil*	1·3	0·3
F ...	28·8	0·8	2·4	2·6	0·3	1·3	0·1	1·5	0·8	0·1
G ...	22·6	6·8	2·2	0·9	0·2	1·8	1·1	4·6	0·2	0·2
H ...	16·4	5·3	0·2	6·4	0·5	1·6	1·2	*Nil*	0·5	0·1
K ...	20·2	0·7	0·3	1·2	0·2	0·5	0·4	*Nil*	0·3	*Nil*

On termination of the dietary surveys discreet attempts were made to assess the approximate income of each family on the basis of the average of the last three years. The average daily income in annas per c.u. in each of the groups of families along with the actual number of consumers in each family, the estimated number of man-value consumption units (c.u.) and other details are shown in Table I. The average daily consumption of the different type of edibles per c.u. in all the nine family groups has been given in Table II. The group symbols suggested in Table I

have been used in Table II and also in the text for the purposes of abbreviation. In some of the groups the pulse intake figures are higher than 6·0 oz.   This is not surprising as Bengal gram flour was consumed in addition to the usual pulse gruel.   The extremely inadequate intake of pulses amongst jute cultivators is compensated for by the unusually high intake of cereals.   The Momins of Santhal Perganas were too poor to purchase adequate amounts of pulses. Though inadequate, the better consumption of milk and milk products as well as fruits and nuts by the Sisbadhia Muslims (G) deserves mention as their cousins (F) in the other part of the same district and belonging to almost the same economic level were found not to consume these protective foods in any appreciable degree.   Consumption of eggs was recorded in groups A and B only.   The cereals consumed consisted of rice, wheat, maize and millets (mainly *marua* or *Eleusine coracana*).   Rice was the most popular cereal and wheat came next in order.   The pulses consisted of red gram, lentil and Bengal gram, though small amounts of black gram, horse gram and lathyrus were eaten.   The non-leafy group of vegetables consisted of potato (except in some of the poorest homes), brinjal, vegetable marrow, cluster beans, bottle gourd, ridge gourd, turnip, cauliflower, etc.   Mustard oil was the main cooking fat.

TABLE III.

Consumption in oz. of different foods per c.u. per diem at different income levels.

Limit of income per c.u. per diem in the family.	Number of families in the group.	Cereals.	Pulses.	Leafy vegetables.	Non-leafy vegetables.	Fats and oils.	Flesh foods.	Milk and milk products.	Fruits and nuts.	Condiments.	Other foods.*	Average income in annas.
Up to 2 annas	226	19·0	3·4	0·7	3·9	0·4	1·1	0·9	0·2	0·6	0·3	1·4
Up to 4 annas	233	21·3	4·8	1·0	3·4	0·6	2·0	1·9	0·7	0·9	0·3	2·9
Up to 8 annas	142	20·7	5·0	0·8	4·1	1·1	2·5	3·5	1·0	1·3	0·5	5·4
Over 8 annas	108	20·3	4·7	1·0	5·7	1·8	3·5	5·8	0·9	1·8	1·0	18·4

* Mainly sugar and jaggery.

In Table II, the families, irrespective of their geographical distribution, have been classified into four income groups in order to study the effect of income on the consumption of the different classes of foods, particularly those of the protective groups.   As the total income of any family does not often convey any idea as regards its purchasing power the families have been classified on the basis of income

per consumption unit per diem in annas. In the absence of suitable cost coefficients for family members of different age and sex, the calorie coefficients suggested by the League of Nations have been used in computing the income per consumption unit (c.u.) per-diem. The noticeable feature in the table is that with increase in income the consumption of almost all classes of food except grain foods and leafy vegetables increased. The consumption of all vegetables was below the desirable level even in the highest income group. One would not expect people of the upper income groups to increase the consumption of grain foods, e.g. cereals and pulses. but the absence of increase in the intake of leafy vegetables once more bring into relief the ignorance of healthy food habits. This particular feature, noticed in the diet of the rural population in the present inquiry, was in keeping with similar findings recorded by·the present author (Mitra, 1940) in dietary surveys of urban families in the industrial city of Jamshedpur. The consumption of milk products and fruits leaves much to be desired. Families with *a daily income of slightly more than one rupee and two annas per consumption unit* could certainly have afforded to include larger amounts of milk and fruit in the diet if healthy food habits were known. Unfortunately. the figures recorded in the present inquiry cannot be compared with the observations made by others, as even in the comprehensive monograph published by Wilson and Widdowson (1942) the Muslim families surveyed have been classed as 'middle class' and 'poor' without any reference to their approximate income.

The recent food shortage and increase in price levels afforded an opportunity of observing how people of the upper-middle class reacted to unusual conditions with reference to their food habits. During the months of February to March 1943 a re-survey of diet was carried out in 114 families of Patna district (group B. Table II) which were surveyed originally during the months of September to November 1939 (*see* Table IV). It was found that fruits and sugar had completely disappeared from the diet. A reduction of 68 per cent was noted in the consumption of milk. Corresponding reductions in the consumption of leafy vegetables, cooking fats and flesh foods were approximately 67, 61 and 54 per cent

TABLE IV.

Re-survey of diets in 114 families (1943).

	Cereals.	Pulses.	Leafy vegetables.	Non-leafy vege-tables.	Fats and oils.	Flesh foods.	Milk and milk products.	Fruits and nuts.	Condiments.	Other foods.
Actuals of 1943 in oz.	19·0	4·1	0·6	5·5	0·7	1·7	1·3	Neg.*	0·9	Nil.
As percentages of 1939 intakes.	105·0	100·1	33·3	94·9	38·9	45·9	31·7	†	50·0	†

* Negligible or less than 0·01 oz.
† Percentages could not be calculated.

respectively.　The cereal quota in the diet increased by a little more than 5 per cent probably to compensate for the loss of calories due to the reduction in intake of protective foods.

TABLE V.

Height in inches and weight in pounds of children.

Age in years.	Boys.			Girls.		
	Number examined.	Height in inches.	Weight in pounds.	Number examined.	Height in inches.	Weight in pounds.
Above 2, but up to 3.	221	32·2	22·8	174	31·7	22·3
Up to 4 ...	293	35·8	27·5	189	35·1	26·8
Up to 5 ...	354	39·1	31·1	239	39·0	30·9
Up to 6 ...	444	41·4	34·3	230	41·4	33·1
Up to 7 ...	440	43·7	38·0	236	43·5	36·9
Up to 8 ...	381	45·6	40·9	168	45·3	40·0
Up to 9 ...	491	46·9	43·4	189	46·6	42·3
Up to 10 ...	583	48·6	47·0	150	48·1	46·0
Up to 11 ...	516	50·8	51·1	109	50·0	51·0
Up to 12 ...	497	52·3	56·6	72	51·4	55·3
Up to 13 ...	402	54·7	62·9	23	53·6	62·4
Up to 14 ...	361	57·2	65·0	...	...	...

NUTRITION SURVEY.

In each of the nine localities (A to K, Table I) where dietary surveys were carried out a large number of children were examined to discover their state of nutrition and rated as 'good', 'fair' and 'poor' by naked-eye examination, in addition to the measurement of their height in inches and weight in pounds.　Investigations for some of the frank signs of deficiency, e.g. phrynoderma, xerophthalmia and angular stomatitis were also carried out.　All children examined in any particular area were not of precisely the same economic status; the only criterion for selection was that they belonged to Muslim families and were above 2 years of age and up to 14 years.

The average figures for girls were slightly lower than those of the boys. On comparing the figures with those of Muslim boys and girls in wheat-eating areas

in North India as recorded by Wilson and Widdowson (*loc. cit.*) it is found that the Bihar Muslim children are less tall and less heavy in all the age groups from 5 years onwards.

In locality A, 511 children were thus examined. The respective numbers examined in localities with group symbols B, C, D, E, F. G, H and K were 999, 566, 943, 628, 452, 1,161, 663 and 833 respectively. In other words all the localities are well represented and in all 6,766 children consisting of 5.013 boys and 1,753 girls were examined.

The rating of their nutrition as 'good', 'fair' and 'poor' was done by naked-eye examination on the lines detailed in a previous communication from these Laboratories (Mitra, 1940).

Sex.	Good, per cent.	Fair, per cent.	Poor, per cent.
Boys	3·9	66·7	29·4
Girls	6·0	64·5	29·5
All children ...	4·4	66·1	29·4

Though on the average only 29·4 per cent of all children were found to be in a 'poor' state of nutrition yet the percentage of children rated as 'poor' varied in different localities. It was 25·2 per cent in area A. 28·9 per cent in area B, 40·1 per cent in area C, 26·9 per cent in area D, 23·9 per cent in area E, 22·3 per cent in area F. 23·9 per cent in area G, 39·8 per cent in area H and 35·8 per cent in area K.

One of the other indices of nutrition that have been recommended by nutrition workers in India is the percentage incidence of some of the frank signs of deficiency, e.g. phrynoderma, xerophthalmia and angular stomatitis. The percentage of children found to be suffering from such signs were as follows :—

Sex.	Phrynoderma.	Xerophthalmia.	Angular stomatitis.	Any of the signs.
Boys ...	5·9	2·1	6·2	13·1
Girls ...	2·2	0·6	3·8	6·3
All children ...	4·9	1·8	5·6	11·4

Th question that naturally arises is whether in those areas, where there was a larger incidence of ' poor ' nutrition, there was a proportionately greater incidence of these deficiency signs. For this purpose the localities have been classed into 3 zones : (*a*) where the incidence of poor nutrition is up to 25 per cent, (*b*) where the incidence of poor nutrition is above 25 and up to 30 per cent, and (*c*) where the incidence of malnutrition is above 30 per cent. Each zone covered 3 of the localities surveyed and the number examined in each exceeded 2,000 (*see* Table VI). It appears that the percentage incidence of ' poor ' nutrition in children was not correlated with the percentage incidence of deficiency signs.

TABLE VI.

Percentage incidence of deficiency signs in graded zones of ' poor ' nutrition.

Class interval of percentage incidence of poor nutrition.	Group symbol of localities.	Number of children.	PERCENTAGE INCIDENCE OF			
			Phryno- derma.	Xeroph- thalmia.	Angular stomatitis.	Any of the signs.
Up to 25 ...	E, F and G	2,251	1·24	1·24	6·35	8·35
Over 25 ... Up to 30 ...	A, B and D	2,453	8·24	1·30	6·85	14·96
Over 30 ...	C, H and K	2,062	4·99	2·81	3·25	10·38

Knudsen-Schiotz index.—Out of the total number of children the dorsal and median furrow of 3,541 boys and 1,036 girls were examined according to its continuity or tortuosity as detailed by Bigwood (1937). According to this index of malnutrition only 21·6 per cent of the boys and 22·4 per cent of the girls were diagnosed as being in an unsatisfactory state of nutrition, whereas according to naked-eye examination, a higher percentage, e.g. 29·4 per cent of the boys and 29·5 per cent of the girls, were rated as showing ' poor ' nutrition. This finding confirms a previous observation of a similar nature made by the author about 3 years ago (Mitra, 1942), after applying the Knudsen-Schiotz index to a little over 2,000 children.

On further analysis of the data it is found that in the case of boys diagnosed to be in a ' poor ' state of nutrition only 38·6 per cent were selected as ' unsatisfactory ' and in the case of girls the corresponding figure was 49·7 per cent, in other words, about 50 to 60 per cent of the children rated as ' poor ' were not selected by this index. Again, out of the total number of children selected as ' unsatisfactory ' (in other words malnourished) by the Knudsen-Schiotz index 29·4 per cent of the boys and 26·6 per cent of the girls were classed as ' fair ' by the naked-eye rating of nutrition. In the circumstances, the method cannot be recommended as fully satisfactory for the assessment of malnutrition. It must, however, be admitted

that none of the children rated as ' good ' were selected as malnourished by this index.

SUMMARY.

1.   Dietary surveys of 709 Muslim families of different economic and social groups, consisting of 4,789 persons, were carried out in the province of Bihar during the years 1939 to 1943.   With increase in income the consumption of pulses, fats and oils, flesh food, non-leafy vegetables and milk and milk products increased, but the consumption of an important protective food, leafy vegetables remained un-affected.   The intake of milk and vegetables even in the highest income group with an average daily income of more than one rupee and two annas per day per c.u. was not satisfactory.   This state of affairs can be ascribed to ignorance of healthy food habits.   During a re-survey of 114 families during the food shortage of 1943, it was found that intake of protective foods like milk and leafy vegetables was greatly reduced.

2.   In and around the villages where diet surveys were carried out, 6,766 children were examined to assess their state of nutrition.   The incidence of ' poor ' nutrition varied from 23 per cent to 40 per cent.   The integrity or continuity of the dorsal or median furrow (Knudsen-Schiotz index) was not found to be a fully satisfactory index of the state of nutrition.   About 13 per cent of the boys and 6 per cent of the girls were found to be suffering from some of the signs of vitamin deficiency.

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NOTICE.

The following has been received for announcement.

—Editor, I.J.M.R.

1st January, 1947.

BRITISH COMMONWEALTH AND EMPIRE HEALTH AND TUBERCULOSIS CONFERENCE, 1947.

A CONFERENCE on Tuberculosis, arranged by the National Association for the Prevention of Tuberculosis of Great Britain, will be held in London on the 8th. 9th and 10th of July, 1947. The Conference will deal with tuberculosis in all its aspects, but with special reference to the problem as affecting the British Commonwealth, and representatives from all the Dominions and Colonies have been invited.

The sessions will include discussions on Tuberculosis in the British Commonwealth and the Colonial Tuberculosis Services; Sanatorium Design; After-care and Rehabilitation; the Psychology of Tuberculosis; New Discoveries in the Prevention and Treatment of the Disease; and the National Health Service and its Effect on Tuberculosis Schemes.

Plans are being made for overseas guests to see something of the anti-tuberculosis work for which Great Britain is famous. and will include visits to sanatoria. hospitals and clinics. and demonstrations of various kinds. The Conference is open to both doctors and laymen. and fuller particulars can be obtained from the Secretary-General. National Association for the Prevention of Tuberculosis. Tavistock House North, Tavistock Square. London. W. C. 1.

A FEEDING EXPERIMENT ON INDIAN ARMY PIONEER RECRUITS, WITH SPECIAL REFERENCE TO THE RELATIVE VALUE OF MEAT AND MILK IN RATIONS.

I Y

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In another communication, Verma (1946) has described the nutritional status of South Indian pioneer recruits, and has demonstrated a correlation between the degree of muscular development and the incidence of clinical signs of malnutrition. This paper deals with the response of the same men to different diets during the first 24 weeks of recruit training. The work was carried out between May and December 1915, in Harihar (Mysore State).

PLAN OF EXPERIMENT.

Subjects.—A total of 801 newly enlisted South Indian pioneer recruits was studied. Details of their racial composition and initial heights and weights have already been reported (Verma, *loc. cit.*). The groups were built up by taking 15 unselected men from fresh arrivals into the experiment daily during May and June (men who had received army ration for more than 2 weeks previously were, however, rejected). All subjects underwent normal training. Routine anthelmintic treatment was withheld. After 24 weeks on controlled diet. the men were discharged from the experimental list.

Diets.—Three different diets were used :—

A. ' Basic diet '—the normal army scale.

B. 'Meat diet'—similar to basic diet, but the normal issue of meat (2 oz.), tinned fish (1½ oz.) and skim milk powder (⅔ oz.) were replaced by fresh meat only (12 oz. dressed, including bone). .

C. 'Milk diet'—similar to the meat diet, but 12 ounces of meat was replaced by 48 ounces of fresh milk (or equivalent in tinned milk).

Details of quantities and composition of these diets are given in *Appendix I*. In order to check the nutritive values of the actual meals consumed the following method was adopted : One man selected at random was approached immediately after he had been given his share from the cookhouse. The food in his mess tins was removed completely. This was repeated at all four meals during the day. The total daily diet thus collected was analysed. This was repeated from time to time during 24 weeks and it was found that the actual and calculated nutritive values did not differ widely. The total and animal proteins supplied by milk and meat diets were of the same order.

The diets were cooked and served at separate cookhouses and dining halls. Full records were kept of the amounts of different foodstuffs issued to cookhouses and of the weight and nature of all kitchen and plate wastage. Menus were prepared in consultation with the men, so as to provide attractive fare and lessen wastage.

Attendance of the right men at the right dining halls was controlled by keeping an attendance roll and by precautions designed to prevent mistaken identity.

Grouping of subjects.—Subgroups were arranged within each of the three dietary groups in order to test the effect of ferrous sulphate on the blood picture. giving seven experimental groups in all :—

A. 'BASIC GROUP' :

(1) On basic diet.
(2) As (1) *plus* 6 grains $FeSO_4$ daily.
(3) As (1) *plus* 3 grains $FeSO_4$ daily.

B. 'MEAT GROUP' :

(4) On meat diet.
(5) As (4) *plus* 6 grains $FeSO_4$ daily.

C. 'MILK GROUP' :

(6) On milk diet.
(7) As (6) *plus* 6 grains $FeSO_4$ daily.

The subjects were divided equally between these seven groups.

Since one of the main intentions in planning this experiment was to study the effect of the various regimes on the blood picture, subjects were equalized in the subgroups according to hæmoglobin level categories at intervals of 0·5 g. per 100 c.c. of blood. To each hæmoglobin category different sets of random numbers (1 to 7), taken from the tables by Fisher and Yates (1943), were allocated. For each hæmoglobin category restricted random distribution of subjects to the various experimental groups was made in accordance with these numbers. Statistical tests show that this technique successfully randomized other variables, e.g. height, body-weight and deficiency signs, etc., between the experimental groups. (For

further details, *see* the report on the hæmatological data by Hynes. Ishaq and Verma, 1946.)

Records kept for each subject.—

(1) Age, as given by the recruiting officer.

(2) Hæmatological data (*see* Hynes *et al., loc. cit.*).

(3) Weight, measured to the nearest quarter pound on a standardized lever-type machine, before breakfast and after evacuation of the bowels.

(4) Height, measured to the nearest quarter inch against a vertical scale, the subject standing ' at attention ' with heels, buttocks, shoulders and occiput in a vertical line.

(5) Results of a complete clinical examination. special note being made of muscular development and lesions indicative of specific dietary deficiencies.

Each subject was examined when he joined the experiment and thereafter at intervals of 8 weeks, thus providing a set of four observations for each.

Owing to wastage (from illness, desertion, etc.), complete records for all subjects beginning the experiment were not available. It was decided to exclude, from the analysis, records of all subjects who came off the experimental diets for 3 weeks or more at any time. Thus, out of the 801 original subjects, records of 616 men only were utilized for statistical analysis.

In the analysis which follows, only the three dietary groups are considered, since there is no reason to believe that the $FeSO_4$ treatment of subgroups exercised any significant influence on the data reported in this paper.

To save space and assist clarity, only the main findings are reported in the following sections. Notes on the statistical treatment of data are given separately in *Appendix II.*

<div align="center">EFFECTS ON WEIGHT.</div>

Graph 1 presents the curves for mean increase of weight for the 3 dietary groups. In all the three groups, weight increases rapidly at first, then slows down and ceases after about 16 weeks. The algebraic equation : $W = A + Bt + C (t^2 - 5/4)$ was fitted to the four readings of body-weight of each subject by the method of least squares, where W is the weight at any time (*t*) measured from the mid-point of the experiment, A the mean of the four weight readings, B the mean growth rate, and C half of the mean change in growth rate. Table I gives the mean values for B and 2C in each dietary group :—

<div align="center">TABLE I.</div>

<div align="center">*Mean rate of weight increase (B) and mean change in growth rate (2C).*</div>

Group.	Number of subjects.	Mean B (lb./8 weeks).	Mean 2C [lb./(8 weeks)²].
Basic	261	+2·410	−3·119
Meat	178	+2·749	−3·503
Milk	177	+2·193	−3·251

Rate of increase of weight is greatest in the meat group and least in the milk group.

Before coming to any conclusion, it is necessary to consider the effect on the growth rate of initial body-weight, age and initial height as these factors, although distributed at random, were not equalized between different dietary groups. Adjustment for these initial differences was made by analysis of covariance. Table II compares the unadjusted mean growth rate in each group with the growth rate adjusted for differences in initial weight, height and age :—

TABLE II.

Mean rate of weight increase (lb./8 weeks).

Mean B.	Basic group.	Meat group.	Milk group.
Unadjusted ...	2·419	2·749	2·193
Adjusted ...	2·432	2·750	2·173

The adjustment does not cause any change in the relative position of the three dietary groups, though each of the factors was found to influence growth rate significantly. Tests of significance applied to the differences between the growth rate of the three dietary groups yield the following results :—

1. The superiority of the meat group over the milk group is highly significant ($P < 0.001$).

2. The superiority of the meat group over the basic group is doubtfully significant ($0.05 > P > 0.02$).

3. The difference between the basic group and the milk group is not significant.

Note.—The recorded ages of subjects were doubtless inaccurate in individual cases, since it is probable that many men overstated their ages (to evade the minimum official age for enlistment, which is 18 years) and others did not know their ages exactly. We, therefore, used height increase in 24 weeks as a measure of adolescence in place of age. To test the assumption that increase of height would be a measure of adolescence, we correlated the stated age of each subject with the amount of height increase during the experimental period of 24 weeks. Six hundred and sixteen pairs of readings gave a correlation coefficient of -0.1, which is significant. This justifies our using the increase of height during the 24 weeks in place of stated ages for adjustment of growth rate in the analysis of covariance.

EFFECT OF AGE.

The subjects in each dietary group were divided into two age categories : (*a*) those below 25 years (probably still growing), and (*b*) 25 years old or older

(growth probably ceased). Table III shows the mean rates of weight increase for each age category :—

TABLE III.

Mean growth rate (lb./8 weeks) according to age and diet.

Age group.	Basic group.	Meat group.	Milk group.
Below 25 years ...	2·437 (219)	2·737 (149)	2·107 (156)
25 years or older ...	2·324 (42)	2·809 (29)	2·833 (21)

Figures in brackets indicate numbers in each group.

A reduced analysis of variance (after adjustment by analysis of covariance) shows that only in the milk group does the growth rate of the two age categories differ significantly. This result indicates that the basic and meat diets provide an equally good stimulus for weight increase, irrespective of age, but the milk diet is relatively inferior for younger subjects who are still growing. It also appears that both age categories were considerably undernourished since subjects whose normal growth had ceased put on weight equally with those who are still of a 'growing' age.

Effect of state of nutrition.—Subjects were divided into the following two categories in accordance with data obtained at the first clinical examination : (a) those with signs indicative of specific deficiencies, and (b) those without such signs. Table IV shows the unadjusted mean growth rates for each category and dietary group :—

TABLE IV.

Mean growth rates (lb./8 weeks) according to state of nutrition.

Nutritional category.	Basic group.	Meat group.	Milk group.
Subjects with deficiency signs ...	2·672 (148)	2·939 (95)	2·248 (101)
Subjects without deficiency signs	2·088 (113)	2·531 (83)	2·121 (76)

Figures in brackets indicate numbers in each group.

It will be seen that the 'deficient' subjects had a higher growth rate than the 'non-deficient' subjects. The differences are significant in the meat and basic

diet groups when adjustments are made for initial height, body-weight and height increase. In the milk group, the difference is not significant even when a breakdown by age categories is made. For all diet groups taken together, the difference is also significant ($P < 0.01$).

Comparison of the mean growth rates for each category as between the various dietary groups indicate that for the 'deficient' subjects, the meat diet is significantly superior to the milk diet, but not to the basic diet, the differences for 'non-deficient' subjects are not significant.

EFFECTS ON HEIGHT.

Two hundred and seventy-six subjects out of the total of 616 showed a measurable increase of height during the experimental period ranging from 0·5 to 1·5 inches. The number of subjects showing an increase of height and average extent of increase for each group is shown in Table V :—

TABLE V.

Group.	Total subjects.	Number showing height increase.	Mean increase (inches) for subjects showing increase in height.
Basic ...	261	116	0·621
Meat ...	178	76	0·625
Milk ...	177	84	0·631

χ-squared test shows that the proportion of subjects showing an increase of height in each group does not vary significantly. The mean increases of height for each group are also not significantly different as seen by analysis of variance. The diets over a short space of 24 weeks, therefore, do not appear to affect differentially either the numbers showing increase in height or the extent of height increase.

NUTRITIONAL STATUS.

Some of the difficulties of recording objective clinical data in an experiment of this nature have already been noted (Thomson, Verma and Dilwali, 1946). In this experiment, careful records were kept of the condition of skin, eyes, mouth and genitals at each 8-weekly examination. Table VI has been constructed to

show the incidence of certain reasonably well-defined conditions at the beginning and at the end of the feeding period:—

TABLE VI.

Number of subjects showing certain clinical signs.

Clinical sign.	BASIC GROUP (261 SUBJECTS).		MEAT GROUP (178 SUBJECTS).		MILK GROUP (177 SUBJECTS).	
	Beginning.	End.	Beginning.	End.	Beginning.	End.
Dry skin ...	187	59	111	36	124	34
Rough skin ...	130	61	88	34	89	27
Phrynoderma ...	*Nil*	*Nil*	2	*Nil*	2	1
Xerosis conjunctivæ ...	46	22	31	13	35	18
Angular stomatitis ...	6	*Nil*	3	*Nil*	3	*Nil*
Fissured tongue ...	12	4	8	1	5	2

χ-squared tests show that the proportions of subjects showing these signs do not differ significantly between groups either at the beginning or at the end of the experiment. Our clinical impression is that the severity of the lesions was likewise similar in each group. The time taken for the disappearance of the various signs did not vary significantly in the different groups. There was, however, a distinct clinical impression that subjects in the milk group had smoother and glossier skins at the end of the 24 weeks.

DISCUSSION.

In a previous feeding experiment on Indian recruits it was found that the gain of weight produced by a basic diet similar to that used in the present experiment was not accelerated by the addition of 16 ounces of milk (Thomson *et al.. loc. cit.*). The analysis of the data reported in this paper indicate that when 12 ounces of fresh mutton (meat diet) or 48 ounces of fresh milk (milk diet) or 2 ounces of fresh mutton plus 1½ ounces of tinned fish plus ⅜ ounce of skim milk powder (basic diet) are added to an otherwise constant diet conforming to a generally satisfactory

nutritional standard (see *Appendix I*), meat diet produces a weight gain which is significantly greater than that produced by the milk diet or the basic diet in subjects showing deficiency signs. On the other hand, we have the impression on purely clinical evidence that the milk alone caused a relatively greater improvement in skin texture.

The effect of meat on the weight gains of our subjects was somewhat unexpected. It is not due to a greater calorie intake on the part of the meat group, and, therefore, seems to be due to some nutritional quality inherent in meat. In the absence of definite evidence, we can only speculate on the nature of this nutritional quality. It is generally accepted that the proteins of milk have exceptionally good growth-promoting qualities (as would be expected in a foodstuff which forms the sole source of nourishment for new-born animals). While we have not attempted to review the literature in detail, there does not seem to be any conclusive evidence that the proteins of meat possess growth-promoting qualities superior to those of milk. (It should be remembered that in all the diets under consideration, an ample supply of protein and a high proportion of animal protein—for Indian diets—was available.) It is possible that the effect of meat was not due to protein but to a stimulating effect on metabolism generally. Colour is lent to this view by the finding that the meat diet was also superior to the milk and basic diets in respect of hæmoglobin regeneration (Hynes *et al., loc. cit.*), and by the work of Whipple and his colleagues on the rôle of different foodstuffs in hæmoglobin regeneration (Whipple, 1942).

Our finding that subjects below 25 years of age on the milk diet showed a rate of growth inferior to that shown by meat group and also of older men in the milk group is difficult to explain and would appear to require experimental confirmation. Another somewhat unexpected finding is that the milk diet was inferior to both the meat and the basic diets for production of weight increase among subjects with definite clinical evidence of specific nutritional deficiencies.

The present finding, that meat has a value superior to that of milk for promoting increase of weight, together with that of Hynes *et al. (loc. cit.)* that meat has special value in blood regeneration, requires careful consideration and, if possible, experimental confirmation, in connection with nutritional policy in India. It is believed that this is the first experimental evidence derived from work on human beings, that a lacto-vegetarian or semi-vegetarian dietary may be intrinsically inferior to a meat dietary. The implications in respect of a predominantly vegetarian population like that of India do not require stressing.

Thomson *et al. (loc. cit.)* have stressed the value of really nutritious diet in the physical regeneration of undernourished Indians recruited into the army. The work now reported confirms this view. The South Indian recruits dealt with are amongst the smallest and most illnourished recruits which the army has ever accepted. With proper feeding and a healthy environment, they can be and are being transformed into reasonably useful soldiers who can undertake the strenuous work required of pioneers. The physical disabilities imposed on them by continued under- and malnutrition during childhood and adolescence can by no means be overcome entirely by good feeding when normal growth has practically ceased; but if better recruits are not available, it pays to provide the best diet that can be provided. According to our findings. meat should form a substantial part of such

a diet. In the case of South Indian recruits, there is no difficulty in persuading them to eat 12 ounces of meat daily, and this would probably be true of all classes except those whose religion imposes a definite ban on the consumption of meat. This is stressed, since it is often stated that the majority of Indians would not eat meat even if it were provided.

SUMMARY.

1. A feeding experiment was carried out at an Indian Army Recruit Training Centre over a period of 24 weeks. The methods and diets used are described.

2. All the three diets (basic, meat and milk) caused a gain in weight of the recruits which, however, was highest on the meat diet and lowest on the milk diet.

3. All subjects showing specific nutritional deficiency signs gained weight more rapidly than the subjects not showing those signs. Meat diet was significantly superior to milk diet for 'deficient' cases. Subjects without specific nutritional deficiencies put on weight equally rapidly on all the three diets.

4. All the three diets produced a marked clinical improvement. It is believed that milk accelerated clinical improvement which was manifested in an improvement of skin texture.

5. No diet affected the increase in height of the recruits differentially.

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APPENDIX I.

A—Scales of diets as given and consumed.

Foodstuffs.	BASIC.		MEAT.		MILK.	
	Scale as given (in ounces).	Scale as actually consumed (in ounces).	Scale as given (in ounces).	Scale as actually consumed (in ounces).	Scale as given (in ounces).	Scale as actually consumed (in ounces)
Cereals :						
Rice	16·0	13·2	16·0	12·4	16·0	12·7
Atta	8·0	7·4	8·0	7·35	8·0	7·3
Pulses :						
Dhal	4·5	3·6	4·5	3·3	4·5	3·2
Dairy produce :						
Ghee	2·75	2·55	2·75	2·50	2·75	2·55
Skim milk powder ...	0·375	0·375	*Nil*	*Nil*	*Nil*	*Nil*
Milk	6·0	6·0	6·0	6·0	54·0	51·0
Meat and fish :						
Meat	2·0	1·6	12·0	9·8	*Nil*	*Nil*
Fish, tinned ...	1·5	1·4	*Nil*	*Nil*	*Nil*	*Nil*
Vegetables and fruit :						
Vegetables, fresh ...	6·0	4·8	6·0	4·7	6·0	4·6
Onions	2·0	1·8	2·0	1·7	2·0	1·6
Potatoes ...	4·0	3·7	4·0	3·6	4·0	3·6
Fruit, fresh ...	2·0	1·55	2·0	1·4	2·0	1·5
Miscellaneous :						
Sugar	3·25	3·25	3·25	3·25	3·25	3·25
Salt	0·5	0·5	0·5	0·5	0·5	0·5
Condiment powder ...	0·57	0·47	0·57	0·47	0·57	0·47
Tea	0·33	0·23	0·33	0·23	0·33	0·23

B—Chemical composition of the diets as consumed.

Nutrients.	Basic.	Meat.	Milk.
Calories	3,790	3,980	4,330
Total proteins (g.) ...	102·6	121·5	119·9
Animal protein (g.) ...	23·8	46·5	46·0
Carbohydrates (g.) ...	636·8	617·4	676·3
Fats (g.) ...	93·1	114·0	128·7
Iron (mg.)	40·5	42·5	38·0
Calcium (g.) ...	0·822	0·625	2·0
Vitamin A (I. U.) ...	2,000	1,900	3,400
Vitamin B₁ (mg.) ...	2·5	2·66	2·450
Riboflavin (mg.) ...	1·40	1·54	3·0
Nicotinic acid (mg.) ...	25·0	37·0	19·0
Vitamin C (mg.) ...	63·0	58·5	57·0

APPENDIX II.

Brief notes on statistical analysis.

Design and general analysis.—In the design of this experiment, which was similar to randomized block type, the initial hæmoglobin level was equalized over dietary subgroups (blocks) which were built up equally rapidly in point of time. Statistically more frequent readings of weight of the subjects would have been desirable, but due to administrative and other difficulties this was not possible. For each subject only four body-weight readings taken at 8-week intervals were available, and as such there was not much point in searching for a suitable degree

GRAPH 1.

Increase of weight for the three dietary groups.

of the polynomial used for growth curve. The mean rate of weight-increase (B) and mean change in rate of weight increase (2C) were obtained with the help of a second degree orthogonal polynomial. These parameters were treated in the analysis as observations. As expected from the shape of growth curves in Graph 1, the values for B were mostly positive and those for C mostly negative. The distribution of B and 2C is discussed in *Appendix III.*

The mean B, set out in Table I of the text, is the arithmetic mean of the values of B in each group. Algebraically these means are the same as would be obtained from the growth curves fitted to the respective mean of the four body-weight readings of the subjects in each diet group. Analyses of variance were done for B and 2C. The differences between diet groups for B were significant at 1 per cent, but not for 2C ($0.25 > P > 0.10$).

Comparison of rates of weight increase in the three diet groups.—Adjustments were made to mean B and mean 2C by allowing for differences in initial height, initial body-weight, and increase in height during experimental period of 24 weeks with the help of analysis of covariance. The partial regression coefficients of initial height and weight on rate of weight increase were highly significant and were respectively $+0.3241$ and -0.0575, while the regression coefficient of the amount of increase in height on rate of weight increase was of doubtful significance ($0.05 > P > 0.02$), its value being $+0.1837$. The reduced analysis of variance for rate of weight increase was :—

	d.f.	s.s.	V	F	P
Between diet groups	2	29·5829	14·7915	5·97	Less than 0·005
Within diet groups	610	1,511·3094	2·4775		
	612	1,540·8923			

The analysis of covariance decreased the error variance by 13 per cent. The 'between groups' significance level increased from $P = 0.01$ to a P less than 0.005. The differences among adjusted means were tested by t-tests, using the reduced 'within group' variance as error variance.

Comparison of mean change in rate of weight increase (2C) in diet groups.—The mean falling off in rates of weight increase in the three diet groups did not differ significantly from one another, even after the adjustment for initial height, weight, and height increase were carried out. The reduced analysis of variance for change in rates of weight increase (2C) was :—

	d.f.	s.s.	V	F	P
Between diet groups	2	13·8454	6·9227	1·456	$0.25 > P > 0.10$
Within diet groups	610	2,899·3644	4·7531		
	612	2,913·2098			

The partial regression coefficient of initial height on 2C was highly significant ($b_1 = -0.3348$) ; of initial weight on 2C was also highly significant ($b_2 = +0.0504$) and of height increase on 2C was in doubtful region of significance ($0.05 > P > 0.02$) and $b_3 = +0.2862$.

Analysis of rate of weight increase according to age and diet.—The inter-group differences among mean rates of weight increase being statistically significant, comparisons for age categories were made within each diet group. The reduced analysis of variance for different diet groups after adjustment for the three factors were :—

	d.f.	s.s.	V	F	P
	For basic group.				
Between age categories	1	1·8271			
Within age categories	256	656·2763	2·5636		
	257	658·1034			
	For meat group.				
Between age categories	1	0·0351			
Within age categories	173	419·3188	2·4238		
	174	419·3539			
	For milk group.				
Between age categories	1	37·9971		17·388	Less than
Within age categories	172	375·8538	2·1852		0·005
	173	413·8509			

Analysis of rate of weight increase according to state of nutrition.—Answers were sought to the following questions :—

(*i*) Did initial malnutrition affect the subsequent growth rate ?

(*ii*) Which diet was better for subjects showing specific deficiency signs ?

As diet groups showed significant differences in rates of weight increase a comparison between each category of state of nutrition was made within each diet group.

The reduced analysis of variance for the three diet groups divided according to specific deficiency signs were as under :—

	d.f.	s.s.	V	F	P
	For basic group.				
Between specific deficiency signs categories.	1	21·3344		8·577	0·005
Within categories	256	636·7689	2·4874		
	257	658·1033			
	For meat group.				
Between specific deficiency signs categories.	1	10·2520		4·335	Between 0·05
Within categories	173	409·1019	2·3647		and 0·025
	174	419·3539			

of the polynomial used for growth curve. The mean rate of weight·increase (B) and mean change in rate of weight increase (2C) were obtained with the help of a second degree orthogonal polynomial. These parameters were treated in the analysis as observations. As expected from the shape of growth curves in Graph 1, the values for B were mostly positive and those for C mostly negative. The distribution of B and 2C is discussed in *Appendix III.*

The mean B, set out in Table I of the text, is the arithmetic mean of the values of B in each group. Algebraically these means are the same as would be obtained from the growth curves fitted to the respective mean of the four body-weight readings of the subjects in each diet group. Analyses of variance were done for B and 2C. The differences between diet groups for B were significant at 1 per cent. but not for 2C $(0.25 > P > 0.10)$.

Comparison of rates of weight increase in the three diet groups.—Adjustments were made to mean B and mean 2C by allowing for differences in initial height, initial body-weight, and increase in height during experimental period of 24 weeks with the help of analysis of covariance. The partial regression coefficients of initial height and weight on rate of weight increase were highly significant and were respectively $+0.3241$ and -0.0575, while the regression coefficient of the amount of increase in height on rate of weight increase was of doubtful significance $(0.05 > P > 0.02)$, its value being $+0.1837$. The reduced analysis of variance for rate of weight increase was :—

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Analysis of rate of weight increase according to age and diet.—The inter-group differences among mean rates of weight increase being statistically significant, comparisons for age categories were made within each diet group. The reduced analysis of variance for different diet groups after adjustment for the three factors were :—

For basic group.

	d.f.	s.s.	V	F	P
Between age categories	1	1·8271			
Within age categories	256	656·2763	2·5636		
	257	658·1034			

For meat group.

	d.f.	s.s.	V	F	P
Between age categories	1	0·0351			
Within age categories	173	419·3188	2·4238		
	174	419·3539			

For milk group.

	d.f.	s.s.	V	F	P
Between age categories	1	37·9971		17·388	Less than 0·005
Within age categories	172	375·8538	2·1852		
	173	413·8509			

Analysis of rate of weight increase according to state of nutrition.—Answers were sought to the following questions :—

(*i*) Did initial malnutrition affect the subsequent growth rate ?

(*ii*) Which diet was better for subjects showing specific deficiency signs ?

As diet groups showed significant differences in rates of weight increase a comparison between each category of state of nutrition was made within each diet group.

The reduced analysis of variance for the three diet groups divided according to specific deficiency signs were as under :—

For basic group.

	d.f.	s.s.	V	F	P
Between specific deficiency signs categories.	1	21·3344		8·577	0·005
Within categories	256	636·7689	2·4874		
	257	658·1033			

For meat group.

	d.f.	s.s.	V	F	P
Between specific deficiency signs categories.	1	10·2520		4·335	Between 0·05 and 0·025
Within categories	173	409·1019	2·3647		
	174	419·3539			

For milk group.

	d.f.	s.s.	V	F	P
Between specific deficiency signs categories.	1	0·0240			
Within categories	172	413·8269	2·4060		
	173	413·8509			

Here 'within variance' is significantly greater than 'between variance'. As stated previously the subjects below 25 years in age are significantly different in rate of weight increase from the subjects 25 years and older, so the comparison made above is vitiated. The milk group was, therefore, further divided into two age categories (described in the text) and within each age category comparison of rates of weight increase for deficient versus non-deficient subjects was made. In none of the two age categories was the rate of weight increase for the 'deficient' subjects significantly different from those of the 'non-deficient' subjects.

The probabilities of significance of the three diet groups for 'deficient' versus 'non-deficient' categories were combined by the usual χ-squared technique which gave an overall probability less than 0·01. This shows that the 'deficient' subjects had significantly higher rate of weight increase than the 'non-deficient' subjects.

In order to answer the second question the mean rates of weight increase of the 'deficient' subjects for three diet groups were compared. The reduced analysis of variance for 'deficient' subjects in the three diet groups was :—

	d.f.	s.s.	V	F	P
Between diet groups	2	36·2798	18·1399	7·82	On the average
Within diet groups ...	338	783·8202	2·3190		of 0·005
	340	820·1000			

The adjusted mean rates of weight increase for 'deficient' subjects of the three diet groups were respectively 3·001 lb., 2·154 lb. and 2·696 lb./8 weeks for meat, milk and basic groups. The t-tests with 'within variance' as error showed that for both meat and basic groups the mean rate of weight increase was significantly greater than that for the milk group (P less than 0·01). The mean rates of weight increase for meat and basic groups, however, did not differ significantly from each other.

For 'non-deficient subjects' the reduced analysis of variance was :—

	d.f.	s.s.	V	F	P
Between diet groups	2	6·7938	3·3969	1·369	Between 0·5
Within diet groups	266	659·7022	2·4804		and 0·25
	268	666·5860			

APPENDIX III.

Distribution of B and 2C.—It is proposed to discuss here the distribution of rates of weight increase (B) and changes in rates of weight increase (2C). The usual practice has been to assume them to be distributed normally.

This is a problem of determining a suitable distribution function empirically when no *a priori* theoretical formula is known. In such cases the natural course is, when we know nothing about the nature of the distribution in advance to assume that the distribution function has expectations equal to the moments of the data. The assumption though not accurate is probably as near the truth as any we can make. In searching for a suitable type, the measures of asymmetry and flatness have been found of considerable assistance. In addition to these characteristics, there are a number of others which are useful in sorting out the type of curve which is most suitable for a particular set of data. These new characteristics are put variously in different books, e.g. k_1 of Pearson's (1945) tables is minus J of Fry (1928), and k_2 of Pearson's tables is k of Kendall (1943).

The distribution of growth rates (B) for the meat and the milk groups, and of the changes in growth rate (2C) for all the three diet groups were investigated. The various calculated moments, etc., for the three distributions were as follows :—

Distribution of	Numbers.	b_1	b_2	k_2	J or $(-k_1)$.
Meat (B)	178	0·1249	4·1928	0·0490	—IVE
Milk (B)	177	0·1870	2·6073	−0·1106	—IVE
All groups (2C) ...	616	0·0018	2·9435	−0·0112	—IVE

The Gram-Charlier series can also represent any of these types. Histograms are shown in Graph 2 :—

Testing whether or not the distributions are normal.—Assuming that each was normally distributed, normal curves were fitted by estimating mean and standard deviations from the sample and getting expected frequencies for various class intervals and comparing them with the observed numbers by the χ-square method. On this basis all the distributions did not depart significantly from normality.

While testing for normality as in Fisher (1944) by calculating g_1 and g_2 the following results were, however, obtained for the three distributions :—

Distribution.	g_1	g_2	$t\text{-}g_1$	$t\text{-}g_2$
Meat (B)	0·3564	1·2610	1·96*	3·48†
Milk (B)	0·4361	−0·3694	2·39*	1·02
All groups (2C) ...	0·0422	−0·0477	0·4256	0·2424

* Significant at 5 per cent.   † Significant at 1 per cent.

GRAPH 2.

*Histograms for growth rates (Bs) for meat and milk groups;
and for change in growth rates (2Cs) for all groups.*

Meat group
(for growth rates).

Milk group
(for growth rates).

All groups
(for change in growth rates).
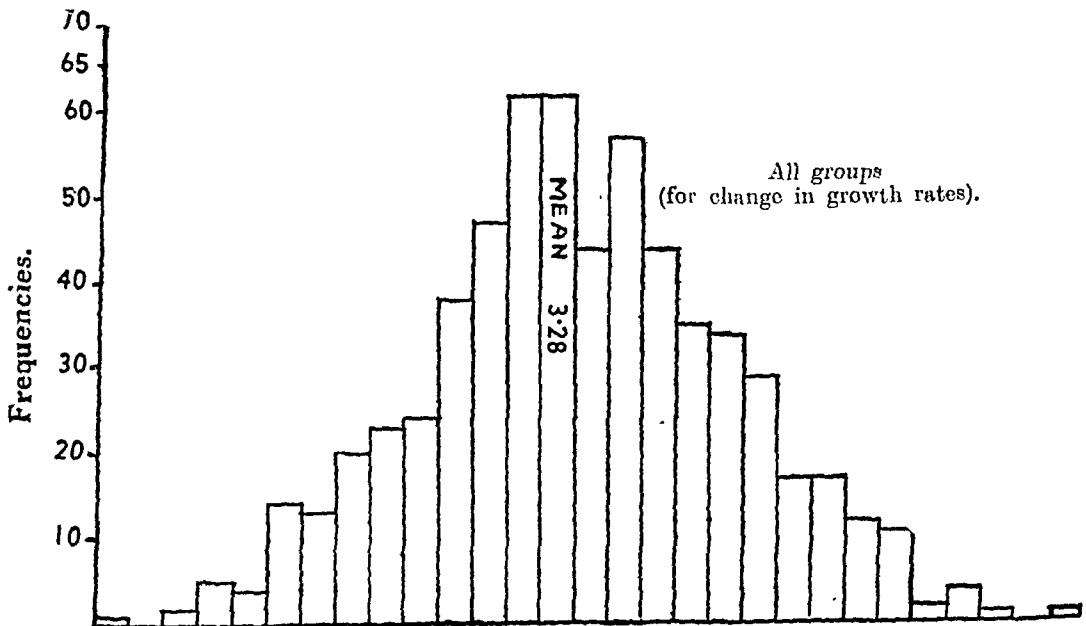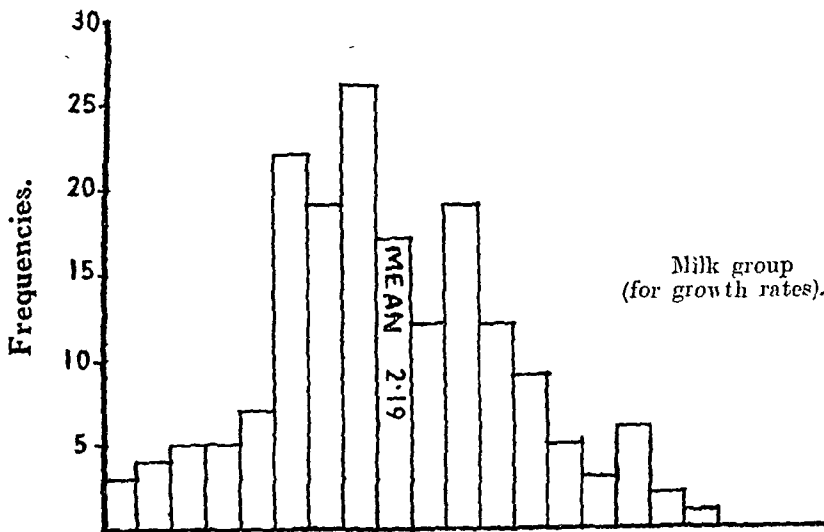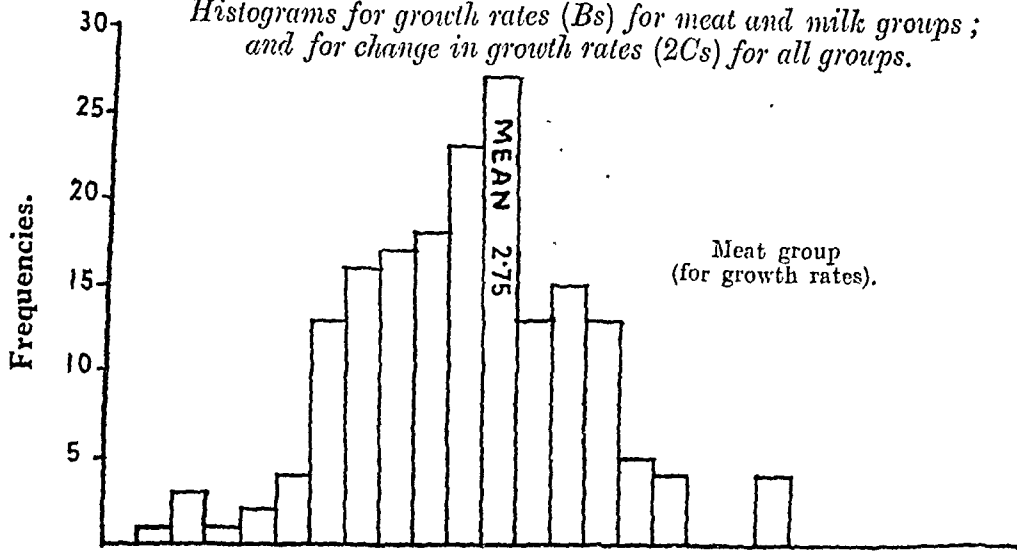
By combining the probabilities of t-g_1 and t-g_2 the verdict from the Table appears that only for values of B in meat group the distribution departs significantly from normality. It should, however, be remembered that sampling distribution of g_2 is very asymmetrical and so the significance of g_2 may at times be very erroneous. The extension of ' Theory of Power of Tests ' to non-parametric case such as the test of normality requires a great deal of further research.

It is hoped to discuss the whole question in some other technical communication, but it appears that the analysis of the paper is not disturbed by these findings.

References used in Appendix III.

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LEVEL OF VITAMIN A IN THE BLOOD AND ITS RELATION TO DARK ADAPTATION AND OTHER OBSERVATIONS.

BY

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I. BLOOD LEVEL OF VITAMIN A AND DARK ADAPTATION.

INTRODUCTION.

SINCE its introduction by Jeans and Zentmire (1934), the biophotometer has been extensively used as a means of detecting latent avitaminosis A. Various workers have tried to improve both the apparatus (Hecht and Shlaer, 1938 ; Hecht and Mandelbaum, 1939) and the technique (Hecht and Mandelbaum, *loc. cit. ;* Dow and Steven, 1941 ; Basu and De, 1941). That impaired dark adaptation is one of the characteristic signs of dietary deficiency in vitamin A is well established (Bodansky and Bodansky, 1941). Certain workers have found the relation between dark adaptation and vitamin A deficiency to exist, though in different degrees (Jeans, Blanchard and Satterwaite, 1941 ; Hecht and Mandelbaum, *loc. cit.*), while others have contradicted it (Yudkin, 1941 ; Dann and Yarborough, 1941, Haig and Patek, 1942). In the present investigation, the biophotometer test and the determination of the blood level of vitamin A were carried out with a view to (1) survey the state of vitamin A in normal persons, (2) investigate the use of the biophotometer in detecting the early stages of vitamin A deficiency and (3) correlate the results of the dark-adaptation test and the vitamin A content of blood.

EXPERIMENTAL.

Technique of the biophotometer test.

The apparatus and the technique employed were similar to those recommended by Jeans, Blanchard and Zentmire (1937) with the following modifications :—

a. (1) The bright light came from above at an angle of 45° and was reflected back from a milk-glass screen.

   (2) In place of the usual optical wedge, use was made of a rheostat and a potentiometer. The resistance of the potentiometer served both to give a dial reading and to vary the intensity of light.

   (3) Against the holes of the quinx plate, photographic plates developed to different intensities were inserted so as to allow corresponding degrees of light transmission. The resistance was so maintained that two spots of light on the left always persisted, serving as fixation points (Hecht and Mandelbaum, *loc. cit.*).

   (4) The diameter of the spots of light was 0·65 cm. so as to subtend nearly an angle of 3° at the eye (Hecht and Mandelbaum, *loc. cit.*).

b. (1) The preliminary adaptation period was dispensed with (Wald, Jaghers and Arminio, 1938) ; instead, the test was repeated immediately after the first test. A recovery period of ten minutes was allowed on each occasion after exposure to bright light for three minutes.

   (2) The exposure to dim light was reduced to a minimum by means of the potentiometer key.

Two readings were taken at each test and it was found that the recovery periods were reproducible with only a slight variation. As most of the subjects examined were either students or members of the staff of the College, who were well acquainted with the theory of the test and the ·working of the scientific apparatus, the learning and intelligence factors in the test were considered to be of a satisfactory standard.

The graph of the readings of the adaptation test of every subject was drawn and compared with the standard graph. It was judged as a whole. It was considered normal if it was above the standard graph or subtended a greater area above it and vice versa. Contrary to the usual practice, the standard graph used was a smooth curve drawn from the readings of a normal person (subject 34) in whom : (*i*) the blood level of vitamin A was the same as the average found in this investigation, (*ii*) the readings were exactly reproducible in both the recovery periods, (*iii*) both the initial and the final readings were neither too low nor high, and (*iv*) vision was normal. This is illustrated in Graph 1.

Vitamin A content of blood.

The method employed for the determination of carotene and vitamin A was that of Kimble (1939a) with modifications as suggested by Haig and Patek (*loc. cit.*) ; Bodansky, Lewis and Lillienfeld (1943) and Caveness, Satterfield and

GRAPH 1.

Plotted readings of dark-adaptation test (recovery period only).

Time in minutes.

- Standard subject.
- Normal subject or above normal.
- Slightly subnormal subject.
- Subnormal subject.
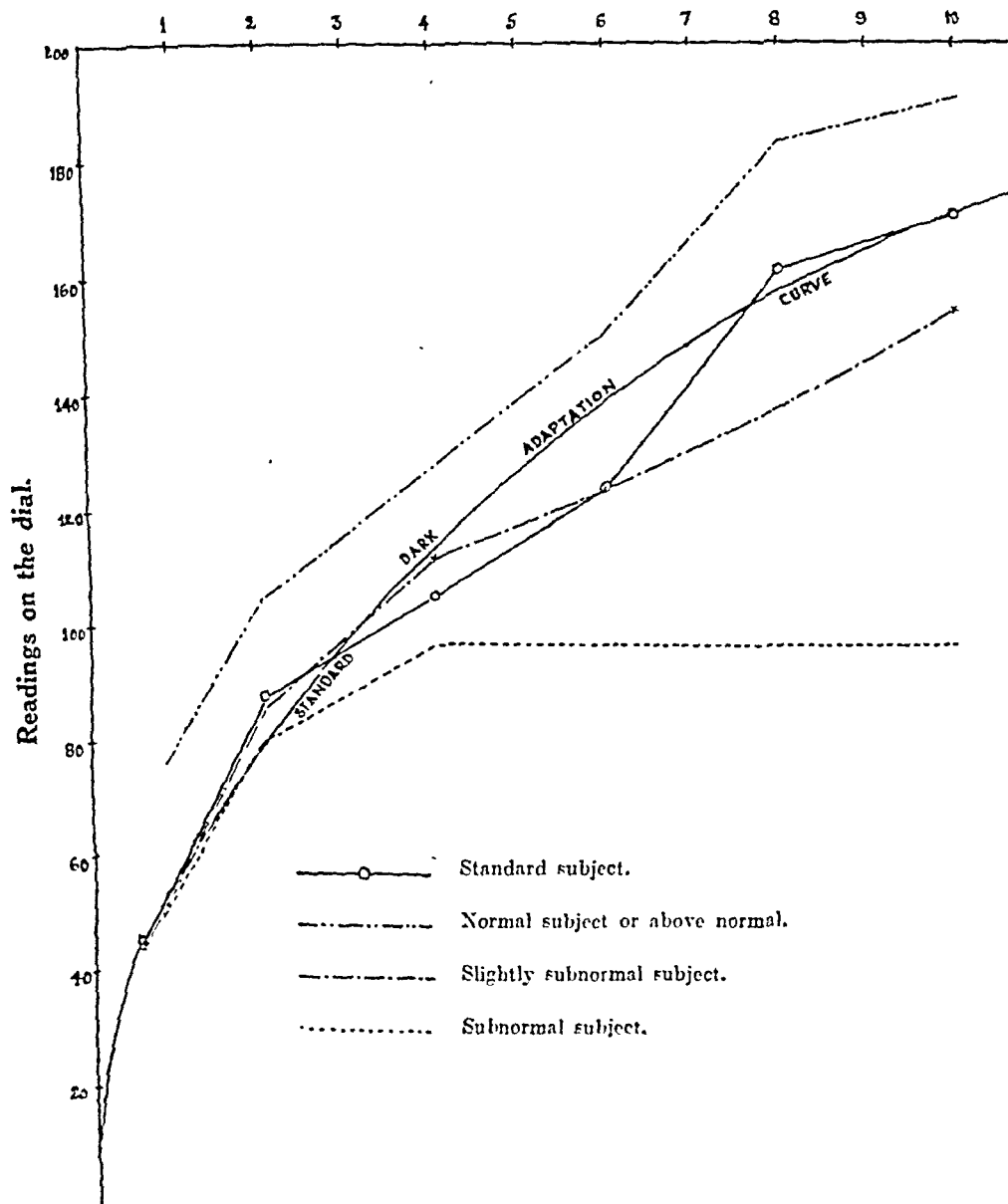
Dann (1941). Instead of deducting the yellow reading of carotene, the separation of carotene from vitamin A was effected by the method of Boyer, Phillips and Smith (1944) and the Blue Units of vitamin A determined separately.

PROCEDURE.

Ten c.c. of blood were drawn by venepuncture, oxalated with two drops of 30 per cent potassium oxalate and centrifuged for 40 minutes. Four c.c. of plasma were pipetted off and run into a 25-c.c. graduated flask and an equal volume of 95 per cent alcohol was added while shaking. The flask was shaken in an ice-bath for 10 minutes, 9·6 c.c. of petroleum ether (boiling range below 67°C.) were added. and the flask shaken for another 10 minutes as before. The contents were then transferred to a 50-c.c. graduated centrifuging tube and centrifuged for 10 minutes. The ethereal layer was completely pipetted off and run into a 100-c.c. conical flask. The petroleum ether was removed under the vacuum of a water suction pump on a water-bath at 70°C. The last traces of petroleum ether were removed by blowing air through once or twice. The residue was dissolved in 3 c.c. anæsthetic chloroform and cooled in an ice-bath. Two determinations of the Blue Value, each with 0·75 c.c. of the solution and an equal volume of antimony trichloride reagent were made on a Lovibond Tintometer. The total Blue Value of vitamin A and carotene in the blood was thus found.

From the remaining 1·5 c.c. solution, chloroform was removed under vacuum and on the water-bath as before. The residue was dissolved in 4 c.c. absolute ethyl alcohol and cooled in an ice-bath. To this 3 c.c. of 0·05 per cent NaCl solution in an ice-bath were added drop by drop while shaking. The contents were ice-cooled for 10 to 15 minutes and then filtered on a Whatman No. 40 filter-paper. The residue was washed with 5 c.c. of a solution of 3 parts 0·05 per cent NaCl and 4 parts absolute alcohol. The carotene was left on the filter-paper. The filtrate was made up to 17 c.c. with distilled water and 6 c.c. of petroleum ether added to it. The contents were shaken for 10 minutes in 25-c.c. graduated flask in an ice-bath and then centrifuged for 10 minutes. The ethereal layer was completely pipetted off and the petroleum ether removed as before. The residue was dissolved in 2 c.c. chloroform and the Blue Value was determined.

In estimating the blood level of vitamin A the carotene was sometimes not completely precipitated but formed a colloidal solution which passed through the filter-paper. This, however, did not affect the result as the precipitate did not re-dissolve when shaken with petroleum ether. Both the ethereal extract and the chloroform solution were colourless after separation or filtration. The method yielded reproducible results.

RESULTS.

The results of the investigation are given in Table I. The values have been expressed in Blue Units as there is no definite conversion factor to express them in International Units. While Leong (1941) has converted Blue Units into International Units by multiplying by the factor 0·6, C. P. values have been converted into I. U. by multiplying by the factor 30 (Heilbron, Jones and Bacharach, 1944). One of the authors also found the same conversion factor in another investigation on fish-liver oil (Ahmad, Ram Chand and Hassan, 1944). This means that 1 B. U. = 1·2 I. U.

TABLE 1

Serial No.	Subject	Diet	Vitamin A + carotene B.U. per 100 c.c. plasma.	Vitamin A B.U. per 100 c.c. plasma.	Vision.	MEAN DIAL READING OF RECOVERY PERIOD AT					
						30 sec.	2 min.	4 min.	6 min.	8 min.	10 min.
1	2	3	4	5	6	7	8	9	10	11	12
	MALES.										
1	M. H.	Mixed.	184	...	...	...	...	...	...	...	...
		"	180	...	...	...	...	...	...	...	...
		"	171	...	...	...	...	...	...	...	...
		"	173	91	...	...	...	...	...	...	...
2	K. R. B.	"	205	56	Corrected with glasses.	55	81	...	122	...	140
3	A. H. A.	"	250	60	...	60	100	114	126	138	162
4	N. A.	"	119	33	...	33	65	76	84	116	120
5	M. N. K.	"	170	147	...	55	95	110	118	131	143
6	M. S.	"	200	123	...	48	90	111	117	133	157
7	K. S.	Vegetarian.	132	110	...	45	95	132	145	150	154
8	G. R. D.	Mixed.	231	100	Corrected with glasses.	60	90	105	121	130	144
9	M. N.	"	181	116	...	60	95	120	133	150	163
10	M. R.	"	220	...	...	51	97	118	133	141	164

TABLE I—*contd.*

Serial No.	Subject.	Diet.	Vitamin A + carotene B.U. per 100 c.c. plasma.	Vitamin A B.U. per 100 c.c. plasma.	Vision.	MEAN DIAL READING OF RECOVERY PERIOD AT					
						30 sec.	2 min.	4 min.	6 min.	8 min.	10 min.
1	2	3	4	5	6	7	8	9	10	11	12
	MALES.										
11	A. H. ...	Mixed.	172	85	...	58	85	106	126	143	166
12	A. L. ...	Vegetarian.	132	84	...	33	73	84	98	112	120
13	A. R. ...	Mixed.	215	...	...	39	72	92	94	96	111
14	K. R. ...	Vegetarian.	187	137	...	44	80	111	124	140	152
15	B. N. B. ...	,,	225	100	...	50	80	93	103	113	128
16	C. B. T. ...	Mixed.	170	...	...	44	80	97	97	97	97
17	M. S. ...	Vegetarian.	170	133	...	50	90	105	119	125	125
18	A. K. ...	Mixed.	195	105	...	35	74	86	108	126	139
19	I. S. G. ...	Vegetarian.	158	80	...	50	97	97	115	121	133
20	M. A. ...	Mixed.	260	89	...	...	...	...	...	...	...
21	A. S. ...	Vegetarian.	181	67	...	74	105	117	143	166	176
22	R. P. K. ...	,,	250	65	...	32	74	87	106	124	151
23	C. R. N. ...	,,	140	50	...	25	79	95	125	140	160
24	K. S. ...	,,	187	45	...	...	...	...	...	...	...
25	M. A. K. ...	Mixed.	120	54	...	60	107	113	135	145	168
26	S. S. M. ...	,,	126	45	...	76	106	134	166	166	170

No.		Diet		(Standard Subject)							
27	M. L. ...	Mixed.	75	40	...	45	84	108	131	146	162
28	H. S. ...	Vegetarian.	111	18	...	50	83	100	104	113	120
29	N. A. ...	Mixed.	120	50	...	...	...	...	...	...	...
30	K. S. ...	Vegetarian.	195	88	...	50	100	114	138	167	184
31	R. S. S. ...	,,	162	...	...	46	75	96	127	145	160
32	S. M. A. ...	Mixed.	125	62	...	47	80	104	135	154	178
33	I. A. S. ...	,,	132	67	Corrected with glasses.	60	90	128	157	175	187
34	V. D. S. O. ...	,,	174	88	...	45	88	106	124	161	170
35	S. M. S. ...	,,	156	91	...	40	83	118	134	...	159
36	A. H. ...	,,	136	81	...	40	85	110	131	163	181
37	A. M. ...	,,	119	44	Corrected with glasses.	30	68	83	105	115	133
38	M. L. ...	,,	110	...	Corrected with glasses.	70	114	139	150	170	174
39	M. S. B. ...	,,	160	89	...	55	100	118	135	159	170
40	W. A. ...	,,	131	83	...	55	100	131	150	168	185
41	W. A. ...	,,	139	50	...	65	110	132	157	170	195
42	B. A. ...	,,	150	85	...	80	113	145	153	178	180
43	A. K. ...	,,	180	...	...	63	104	126	145	167	187
44	A. K. ...	,,	163	89	Corrected with glasses.	66	130	153	189	185	210
45	A. A. N. ...	,,	163	89	...	60	130	143	150	164	171

TABLE I—*concld.*

Serial No.	Subject.	Diet.	Vitamin A + carotene B.U. per 100 c.c. plasma.	Vitamin A B.U. per 100 c.c. plasma.	Vision.	MEAN DIAL READING OF RECOVERY PERIOD AT					
						30 sec.	2 min.	4 min.	6 min.	8 min.	10 min.
1	2	3	4	5	6	7	8	9	10	11	12
MALES.											
46	A.S. ...	Mixed.	150	103	...	84	136	160	179	210	220
47	B.A. ...	,,	178	92	Corrected with glasses.	60	110	135	150	165	168
48	M. H. ...	,,	170	107	...	60	105	135	154	177	204
FEMALES.											
1	U. K. ...	Mixed.	144	80	...	70	97	125	150	161	182
2	S. D. ...	Vegetarian.	222	91	Corrected with glasses.	58	109	127	150	177	189
3	M. B. ...	Mixed.	216	102	...	30	80	94	105	131	139
4	A. K. ...	,,	145	85	...	50	100	...	131	160	180
5	A. K. D. ...	,,	168	86	Corrected with glasses.	23	77	95	114	...	125
6	T. A. ...	,,	192	90	...	60	103	118	176	195	210
7	A. P. K. ...	Vegetarian.	182	40	Corrected with glasses.	38	94	120	139	159	183
8	M. C. ...	Mixed.	170	88	...	58	100	138	170	182	186
9	G. L. ...	,,	126	61	Corrected with glasses.	45	83	107	140	160	184
10	D. K. ...	Vegetarian.	116	71	...	60	90	115	130	156	165
11	I. Q. ...	Mixed.	150	86	...	60	115	135	162	179	189
12	R. K. ...	Vegetarian.	205	97	Corrected with glasses.	60	110	130	150	170	180
13	S. D. ...	,,	108	53	Do.	70	125	166	178	188	200
14	B. K. ...	,,	125	47	...	75	109	144	171	206	226
15	H. B. ...	Mixed.	110	62	Corrected with glasses. Do.	43	111	122	134	180	200

Average value in Blue Units per 100 c.c. plasma.

	Vitamin A + carotene.	Mean deviation.	Vitamin A.	Mean deviation.
Males	168	± 30	91	± 25
Females	158	± 32	76	± 15

The average levels of vitamin A and carotene given above represent the values of a group of college students between the ages of 19 and 23. College students as a class may be regarded as a well-fed group in comparison with the general population. Lower values may, therefore, be expected in the general population where under-nutrition and inadequate diet are not uncommon. The values in this investigation also represent the values of an age group where optimum values may be found.

The average levels of vitamin A and carotene as reported by some of the workers are as follows :—

Vitamin A values per 100 c.c. plasma.

	VITAMIN A VALUES.		CAROTENE VALUES.	
	For males.	For females.	For males.	For females.
Kimble (1939b)	127 I. U.	91 I. U.	...	...
Popper and Steigman (1943) ...	58μ.	47μ.	74μ.	85μ.

A comparison of the values will show that the values found in the present investigation are somewhat lower than those reported by the other workers. This difference may be due to the difference in diets of an average Punjabi and an average American. The diet in the case of the latter is better both in quality and quantity.

An interesting observation is the average value of vitamin A content of blood in females. It was not found to differ greatly from that of the males in this investigation, nor was it found to be much lower than the values reported by Kimble (1939b). Considering the values one is led to the conclusion that the health of the group of females was comparatively better than that of the male group. This is further supported by the results of the dark-adaptation test.

CORRELATION OF VITAMIN A LEVEL AND DARK ADAPTATION.

In this investigation no definite relation has been found to exist between the vitamin A level of blood and results of the dark-adaptation test in normal persons

GRAPH 3.

Plotted readings of dark-adaptation test (recovery period only).
Subject 12   A. L. (male), Sweeper.

Subject 12.

Same subject after 6 da

,,   ,,   5 we

Initial blood level of   . U. p
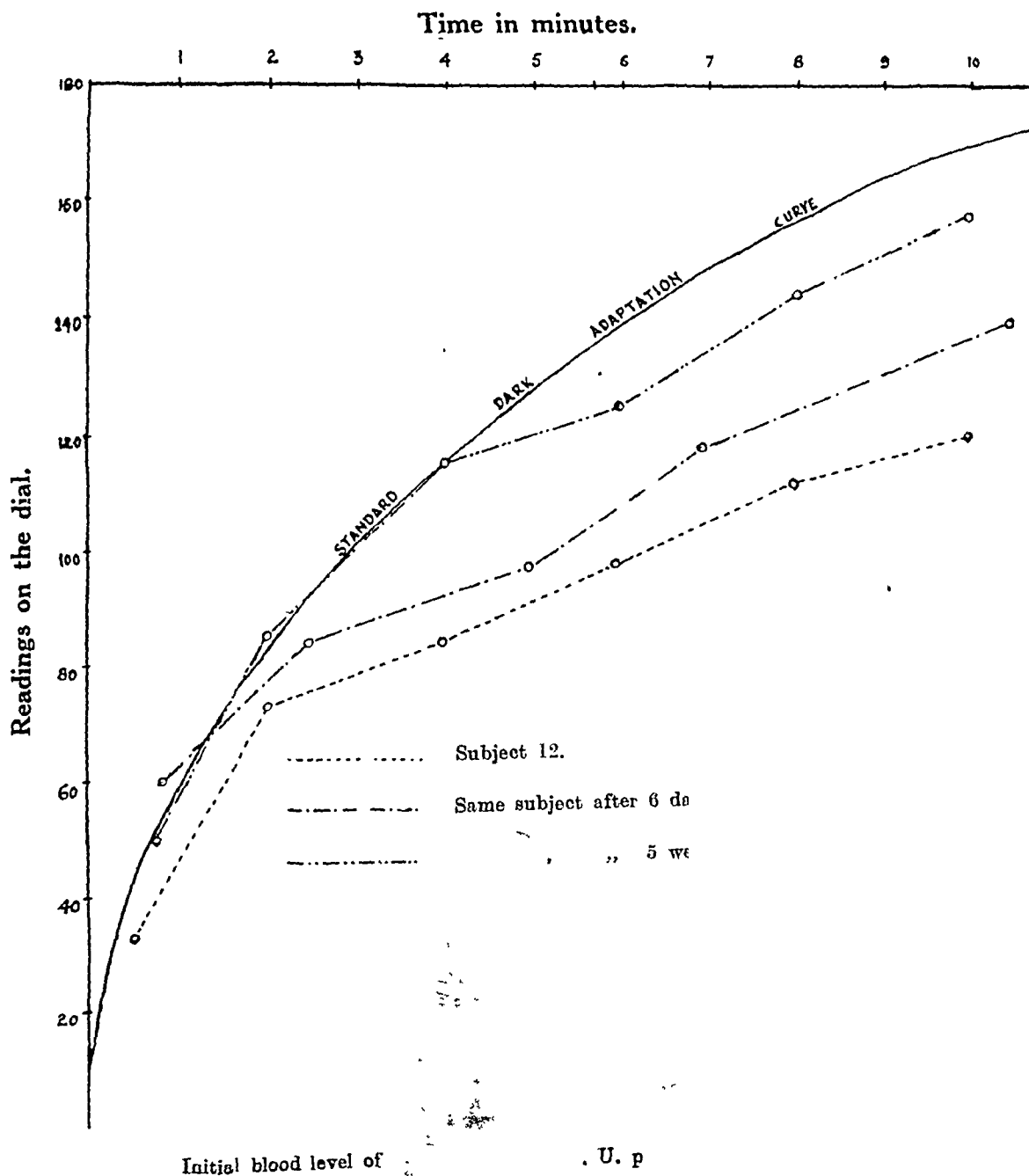
GRAPH 4.

Plotted readings of dark-adaptation test (recovery period only).
Subject 15   B. N. B. (male), College Student.

Time in minutes.
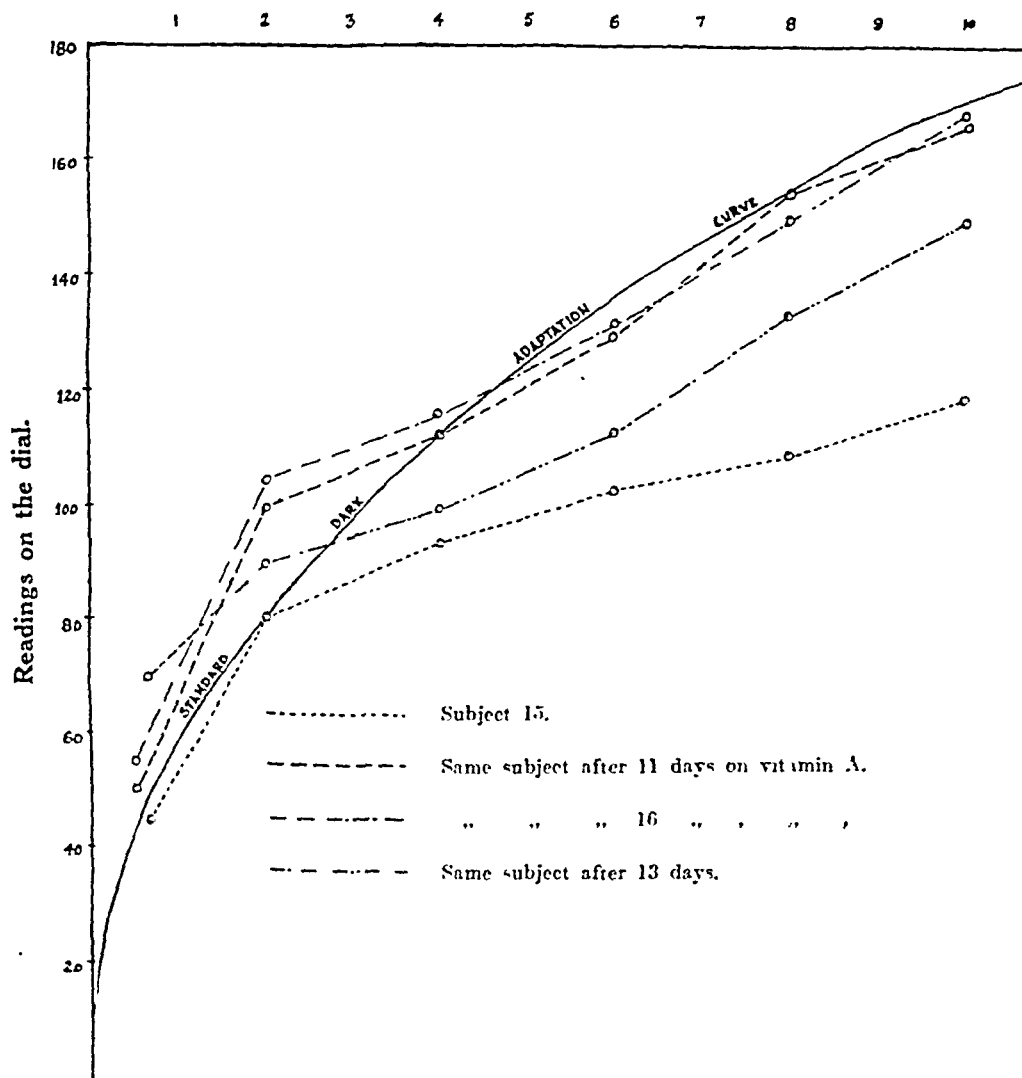
Initial blood level of vitamin A = 100 B. U. per 100 c.c. of plasma.

Subjects 16 (C. B. T.) and 28 (H. S.) besides very poor dark adaptation and a low level of vitamin A also showed other symptoms of vitamin A deficiency. Administration of vitamin A in the first case (C. B. T.) resulted in (1) improvement of dark adaptation, (2) disappearance of other symptoms, and (3) increase in weight. The second subject (H. S.) gained in weight and showed a general improvement in condition. A gain of 7 lb. in weight was also observed in subject 4 (N. A.) whose results are illustrated in Graph 2.

OTHER FACTORS DETERMINING DARK ADAPTATION.

The response to dark adaptation was good in 13 out of 14 subjects suffering from myopia whose error of refraction was corrected by suitable spectacles. In the 14th case it was suspected that his glasses were unsuitable. It is also probable that the results of the dark-adaptation test were complicated by the inclusion of a number of subjects in whom myopia was not detected. Besides other requisites, it must also be ascertained that all persons whose dark adaptation is examined should have normal vision. In this connection it will be of interest to note that three subjects whose blood level of vitamin A was normal but showed poor dark adaptation were found to be myopes when tested. Subject 15 (B. N. B.) is one example.

The authors' findings, therefore, confirm those of other workers that defective vision should be taken into consideration and that the dark-adaptation test is reliable for detecting latent avitaminosis A only if all the persons examined have the same standard of visual acuity.

II. THE RELATIONSHIP BETWEEN THE BLOOD LEVEL OF VITAMIN A AND (1) BLOOD SEDIMENTATION, AND (2) TOTAL VITAMIN A AND CAROTENE.

BLOOD SEDIMENTATION.

Samples of blood drawn from 37 male and 14 female subjects were centrifuged for a constant time (40 minutes) at the same speed in the same centrifuge. It was found that the same volume of blood yielded different volumes of plasma in different individuals and that the greater the yield of plasma the lower were the values for vitamin A estimation. Actual findings are recorded in Table II :—

TABLE II.

Serial number and sex of subject.	Percentage of plasma obtained from constant volume of blood.	Vitamin A B. U./100 c.c. plasma.
MALE.		
1	41	133
2	41	89

TABLE II—*contd.*

Serial number and sex of subject.	Percentage of plasma obtained from constant volume of blood.	Vitamin A B. U./100 c.c. plasma.
MALE.		
3	41	88
4	41·5	147
5	42	88
6	43	107
7	43	89
8	43	85
9	43	56
10	44	105
11	45	103
12	45	84
13	45	65
14	45·5	40
15	47	137
16	47	62
17	47	45
18	47·5	69
19	48	89
20	48	54
21	49	89
22	49	83
23	49	80
24	49	50
25	50	100
26	50	92
27	50	18
28	51	123

TABLE II—*concld.*

Serial number and sex of subject.	Percentage of plasma obtained from constant volume of blood.	Vitamin A B. U./100 c.c. plasma.
MALE.		
29	51	116
30	51	91
31	51	85
32	51	81
33	51	67
34	51	62
35	51	50
36	51	44
37	51	33
FEMALE.		
1	46	88
2	51	102
3	51	90
4	51	86
5	51·5	85
6	52	97
7	53	61
8	55	86
9	55	80
10	55	47
11	56	71
12	57	53
13	58	62
14	59	40

As will be seen from Table II, there is a tendency for lower levels of vitamin A to occur in those samples of blood which yielded a relatively high percentage of plasma from a constant volume of blood. This was particularly noticeable in the

case of female subjects. These findings suggest that a low level of vitamin A is associated with an increased sedimentation rate of the blood and it is possible that by the use of a more accurate method of determining the sedimentation rate of the blood a closer correlation between blood levels of vitamin A and sedimentation rate might be demonstrated.

RATE OF VITAMIN A TO TOTAL VITAMIN A AND CAROTENE.

The percentage of vitamin A in total vitamin A plus carotene was estimated in 40 male and 15 female subjects. The results are recorded in Table III :—

TABLE III.

Serial number and sex of subject.	Percentage of vitamin A in total vitamin A + carotene.	Vitamin A B. U./100 c.c. plasma.
MALE.		
1	91	123
2	78	133
3	73	137
4	69	103
5	63	107
6	63	84
7	63	40
8	62	83
9	60	81
10	58	91
11	57	85
12	55	100
13	55	89
14	54	105
15	54	100
16	54	89
17	54	59
18	53	116
19	51	92

TABLE III—*contd.*

Serial number and sex of subject.	Percentage of vitamin A in total vitamin A + carotene.	Vitamin A B. U./100 c.c. plasma.
MALE.		
20	51	88
21	51	81
22	51	67
23	50	62
24	47	110
25	45·5	85
26	45	88
27	45	84
28	42	50
29	40	100
30	37	67
31	37	44
32	36	50
33	36	50
34	36	45
35	34	89
36	28	69
37	27	33
38	26	65
39	24	45
40	16	18
FEMALE.		
1	61	71
2	58	85
3	57	86
4	56	62

TABLE III—*concld.*

Serial number and sex of subject.	Percentage of vitamin A in total vitamin A + carotene.	Vitamin A B. U./100 c.c. plasma.
FEMALE.		
5	55	80
6	52	88
7	51	86
8	49	53
9	48	61
10	47	102
11	47	97
12	47	90
13	47	91
14	37	97
15	23	40

It might be expected that, in the case of subjects suffering from vitamin A deficiency, carotene would be converted into vitamin A and consequently it would be reasonable to expect that, in such cases, there would be a high $\frac{\text{vitamin A}}{\text{vitamin A} + \text{carotene}}$ ratio. The results of observations recorded in Table III do not, however, fulfil this expectation. These observations were, of course, made on apparently normal subjects. The general tendency was for a decrease in the blood level of vitamin A to be associated with a decrease in the percentage of vitamin A in total vitamin A plus carotene. It would appear, therefore, that, in the subjects examined, a low level of vitamin A in the blood was not due solely to a lack of this factor in the diet but probably also to the inability of the body to convert carotene into vitamin A, or to a combination of these factors.

SUMMARY.

1. The dark-adaptation test was carried out in 48 male and 15 female students of the King Edward Medical College, Lahore, in whom the blood levels of vitamin A and carotene were ascertained. The results of the latter estimations are summarized below :—

Blue Units of vitamin A per 100 c.c. plasma.

	Vitamin A.	Vitamin A + carotene.
Male subjects	91 ± 25	168 ± 30
Female subjects	76 ± 15	158 ± 25

These findings confirm those of Yudkin (*loc. cit.*).

The average critical level of vitamin A in the blood was found to be 60 B. U. per 100 c.c. plasma in the case of male subjects and 50 B. U. in female subjects. In 11 male and 2 female subjects the vitamin A level was below the average critical level.

2. The dark-adaptation test, as carried out in this investigation, was found to be a reliable means of detecting subjects with very low blood levels of vitamin A in the absence of any other symptoms of vitamin A deficiency. No definite correlation was observed between the dark-adaptation tests and normal or slightly subnormal blood levels of vitamin A. All subjects who responded badly to the dark-adaptation test showed a distinctly better response to the dark-adaptation test after the administration of vitamin A.

3. In 14 subjects in whom myopia was corrected with glasses, response to dark adaptation was good. As myopia is prevalent in the type of subjects examined it is necessary to ensure that all subjects have the same level of vision.

4. Some evidence has been adduced to suggest that a low blood level of vitamin A is associated with an increased sedimentation rate of the blood.

5. A decrease in the blood level of vitamin A appears to be associated with a decrease in the percentage of vitamin A in total vitamin A plus carotene.

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THE CAROTENOID PIGMENTS AND THE VITAMIN A ACTIVITY OF INDIAN CARROTS.

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CARROTS (*Daucus carota*) have been the subject of the earliest investigations on carotenoids and have served as an important commercial source of carotene. During the last two decades, a number of workers have studied the carotenoids of this vegetable. It was found that besides β-carotene, the carotenes of carrots consisted of 10 to 20 per cent of α-carotene (Kuhn and Lederer, 1931 ; Wackenroder, 1931 ; Bills and McDonald, 1932 ; Karrer and Walker, 1933, 1934 ; Kuhn and Lederer, 1941). Kuhn and Brockmann (1933*a*, 1933*b*) identified γ-carotene in carrots in addition to α-carotene and β-carotene. Strain (1939) showed that carrots contained still another pigment, δ-carotene, which was found to be identical with a carotene first detected in carrots by van Stolk, Guilbert and Penau (1932). Very recently, a fifth pigment has been shown to occur in carrots by Harper and Zscheile (1945). This they called ζ-carotene. These authors also observed the presence of lycopene in a number of commercial varieties. The presence of two cis-trans isomers of β-carotene in carrots, namely neo-β-carotene U and neo-β-carotene B, has been shown by Kemmerer and Fraps (1945*a*) and Kemmerer, Fraps and Meinke (1945).

Carrots found in the country vary in colour from pale yellow, pink, orange, to deep red. A number of violet-coloured varieties also occur with varying rich-ness of the shade. In the course of investigations on carotenoids of plant foods, it was considered of great interest to investigate in some detail the carotenoids of various varieties of carrots grown in this region. The subject is also of practical

importance since carrots constitute an important item of food, and are used as a commercial source of carotene for fortifying edible fats.

Fourteen different varieties of carrots ranging in colour from deep red to orange, yellow and violet were investigated. Red varieties showed the presence of seven different bands in the chromatograph; orange, six different bands; violet and pink, four different bands; while pale yellow to deep yellow varieties showed from two to six different bands.

METHODS.

The procedure followed for complete extraction of the carotenoid pigments is described in an earlier paper (Sadana and Ahmad, 1946). Alumina* was used as an adsorbant for chromatographic analysis, one particular brand of which was found most suitable, as during grinding it helped to reduce the particle size and it facilitated activation. The material which passed through a 300-mesh sieve was activated by heating at 200°C. in a stream of CO_2 for 2 hours. A concentrated solution of the pigments in petroleum ether was used for chromatographic analysis and the bands were developed by petroleum ether containing 30 per cent benzene. The various bands were cut and eluted by means of petroleum ether containing 2 to 3 per cent ethyl alcohol and the amount of different pigments was determined colorimetrically by reference to a standard curve of β-carotene against 0·04 per cent $K_2Cr_2O_7$. The results are expressed as $\mu g./g.$ in terms of the colour of β-carotene.

A typical chromatograph of red carrots is given (*see* opposite).

On the basis of the biological activity of various carotenoid pigments reported in the literature the vitamin A potency is calculated in I. U./g. according to the following formula :—

Vitamin A activity in I. U./g.

$$= \frac{\mu g.\ \beta\text{-}carotene}{0·6} + \frac{\mu g.\ \gamma\text{-}carotene}{1·2} + \frac{\mu g.\ \alpha\text{-}carotene}{1·2} +$$

$$\frac{\mu g.\ neo\text{-}\beta\text{-}carotene\ B}{1·2}\ (\text{Kemmerer and Fraps, 1943}) +$$

$$\frac{\mu g.\ neo\text{-}\beta\text{-}carotene\ U}{2·4}\ (\text{Kemmerer and Fraps, 1945}) +$$

$$\frac{\mu g.\ neo\text{-}\alpha\text{-}carotene}{0·86}\ (\text{Gillam, el Ridi and Kon, *loc. cit.*}).$$

It was considered that a certain amount of the pigments is liable to be lost during the process of chromatographic separation in spite of all reasonable precautions. Therefore, as a matter of routine, the total pigments were colorimetrically estimated after isolation. At the same time, the sum of individual pigments was determined after the chromatographic separation. The difference between the two figures is regarded as a loss occurring during the experiment. These data for the different varieties examined are shown in Table I.

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* The brand of alumina was obtained from Scientific Supplies (Bengal) Co., Calcutta.

A narrow pale yellow band, xanthophyll	...	...	...	...
A narrow pink band, γ-carotene	...	...	...	...
A narrow yellowish band, neo-β-carotene U	...	...	...	...
A broad red band, β-carotene	...	...	...	...
A brownish yellow band, neo-β-carotene B	...	...	...	...
A wide orange band, α-carotene	...	...	...	...
A narrow yellowish orange band, neo-α-carotene	...	...	...
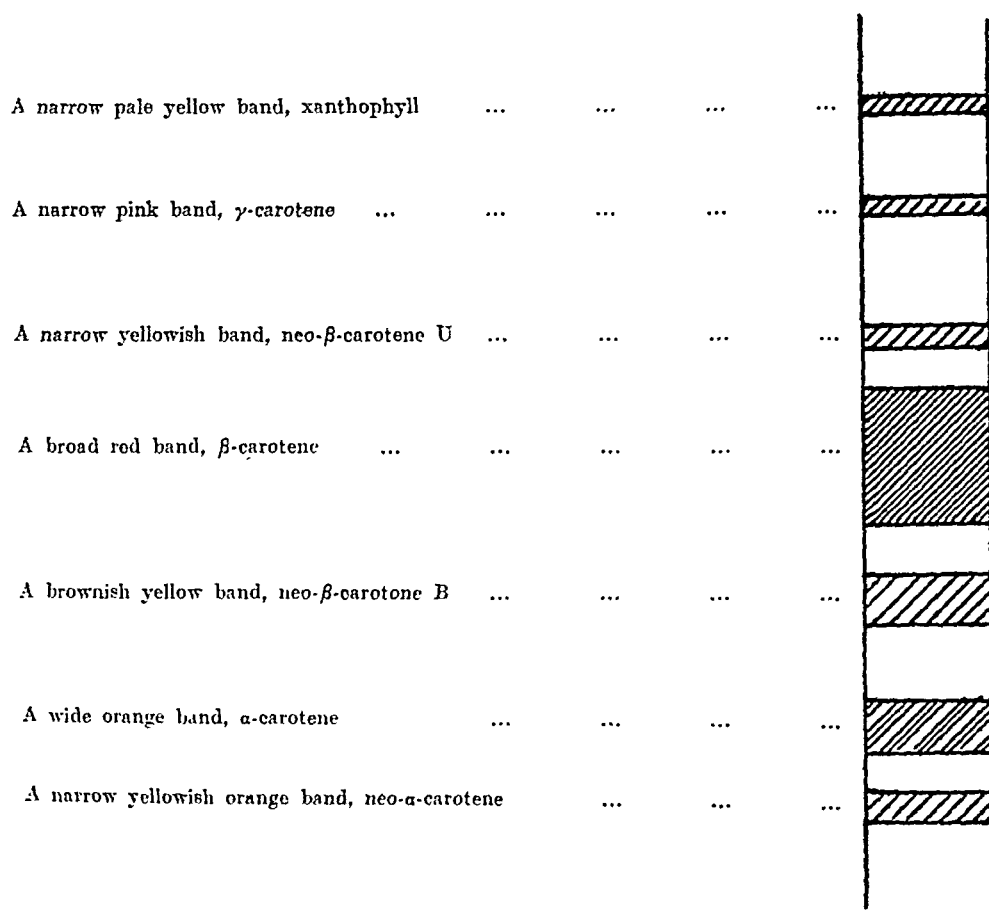
CHROMATOGRAPH.—A typical chromatograph of the pigments of red carrots.

Band No. 1. The pigment was extracted and identified as xanthophyll by mixed chromatography.

„ 2. This was identified as γ-carotene.

„ 3. The pigment of this band represents carotenoid *x* of Kemmerer and Fraps (1943). This pigment was later shown to be identical with neo-β-carotene U described by Polgar and Zechmeister (1942) on the basis of their identical absorption maxima, yielding only a single band on mixed chromatography and the number and ratio of isomers produced on treatment with iodine (Kemmerer and Fraps, 1944).

„ 4. The pigment was isolated and identified as β-carotene by its absorption spectra.

„ 5. It is found to be an isomer of β-carotene and termed pseudo-α-carotene by Gillam and el Ridi (1935, 1936), Zechmeister and Tuzson (1938) and Carter and Gillam (1939). Beadle and Zscheile (1942) preferred to call it neo-β-carotene being derived from its parent substance β-carotene. Polgar and Zechmeister (*loc. cit.*) obtained 12 different isomers by various treatment and called this isomer neo-β-carotene B. The identity of all the three pigments, namely pseudo-α-carotene, neo-β-carotene and neo-β-carotene B, was definitely established by Kemmerer and Fraps (1944).

Band No. 6.   This was identified as a-carotene by means of mixed chromatography, and in view of its optical activity.

,,   7.   From its position on the adsorption column, this pigment is presumably an isomer of a-carotene. It has been named neo-a-carotene by Gillam, el Ridi and Kon (1937).

TABLE I.

Loss of pigments in carrots during chromatographic analysis.

Number.	Colour and description of carrots.		Total pigments in the extract. μg./g.	Sum of individual pigments obtained after chromatographic separation. μg./g.	Loss per cent.
1	2		3	4	5
1	Red colour, grown in Lahore	(a)	112·5	93·5	16·9
		(b)	110·8	95·3	14·0
2	Deep red colour, grown in Delhi ...		150·7	139·6	7·3
3	Red colour, grown in Delhi	(a)	120·5	103·5	14·1
		(b)	100·2	84·5	15·6
4	Light red colour, grown in Delhi ...		70·5	63·1	10·5
5	Deep orange colour, grown in Delhi ...		60·0	50·1	16·5
6	Orange colour, grown in Delhi	(a)	47·3	40·0	15·4
		(b)	34·7	31·4	9·5
7	Pale yellow colour, grown in Delhi ...		2·7	2·2	8·5
8	Light yellow colour, grown in Delhi.	(a)	5·1	4·2	17·6
		(b)	7·4	6·1	17·6
9	Yellow colour, grown in Delhi	(a)	8·5	7·35	13·5
		(b)	10·0	8·28	17·2
10	Deep yellow colour, grown in Delhi ...		15·02	12·52	16·6
11	Pink colour with a yellow core, grown in Delhi.		18·0	15·8	12·2
12	Violet colour with a pale yellow core, grown in Delhi.	(a)	3·0	2·4	20·0
		(b)	5·2	4·13	20·6
13	Violet colour with a yellow core, grown in Delhi.		9·0	7·83	13·0
14	Violet colour with a pale yellow core, grown in Lahore.		3·51	3·19	9·7

(*a*) and (*b*) represent different samples of the same variety.

The loss varies from 7·3 to 16·9 per cent in the case of more highly pigmented varieties and 9·7 to 20·6 per cent in case of less pigmented varieties. In the latter case since the amount of pigments is small, the experimental error is likely to be greater. The order of losses involved in using this technique are, therefore, more correctly represented by the figures obtained in the former case.

Analytical data regarding the carotenoid content of different varieties of carrots is summarized in Table II. Vitamin A potency calculated in accordance with the formula mentioned above are also recorded in Table II.

TABLE II.

The carotenoid pigments of different varieties of carrots and their vitamin A activity.

Number	Variety	Total pigments. μg./g.	Xanthophyll. μg./g.	Xanthophyll. Per cent.	γ-carotene. μg./g.	γ-carotene. Per cent.	Neo-β-carotene U. μg./g.	Neo-β-carotene U. Per cent.	β-carotene. μg./g.	β-carotene. Per cent.
1	2	3	4		5		6		7	
1	Red (Lahore) (a)	93·5	2·1	2·2	0·4	0·4	...	...	80·4*	86·0
	(b)	95·3	2·3	2·4	0·9	0·9	1·4	1·5	78·8*	82·7
2	Deep red (Delhi) (a)	139·6	4·4	3·2	0·7	0·5	1·7	1·2	99·0	70·9
	(b)	103·5	2·9	2·8	2·1	2·0	1·5	1·4	61·9	59·8
3	Red (Delhi) (a)	84·5	2·0	2·4	0·4	0·5	...	...	50·5	60·8
	(b)	63·1	1·8	2·9	...	...	...	...	40·4	64·0
4	Light red (Dolhi) ...	50·1	5·1	10·2	...	...	0·2	0·4	16·0	31·9
5	Deep orange (Delhi) ...	40·0	9·9	24·7	...	...	0·9	2·2	13·0	32·5
6	Orange (Delhi) (a)	31·4	9·2	29·3	...	...	1·0	3·1	4·2	13·3
	(b)	2·2	2·0	90·9	...	...	...	...	0·2	9·1
7	Pale yellow (Delhi)	4·2	3·8	90·4	...	...	0·1	2·4	0·3	7·1
8	Light yellow (Delhi) (a)	6·1	5·7	93·4	...	...	0·1	1·6	0·3	5·0
	(b)	7·35	5·7	77·5	...	...	0·3	4·1	1·3	17·7
9	Yellow (Delhi) ...	8·28	7·1	85·7	0·2	2·4	0·05	0·6	0·83	10·0
10	Deep yellow (Delhi) ...	12·52	9·5	75·9	0·25	2·0	0·13	1·0	2·3	18·4
11	Pink with a yellow core (Delhi) ...	15·8	13·2	83·5	...	...	0·6	3·8	1·8	11·4
12	Violet with a pale yellow core (Delhi) (a)	2·4	2·1	87·5	...	...	0·04	1·7	0·26	10·8
	(b)	4·13	3·5	84·7	...	...	0·07	1·7	0·49	11·9
13	Violet with a yellow core (Dolhi)	7·83	7·0	89·4	...	...	0·08	1·0	0·7	8·9
14	Violet with a pale yellow core (Lahore).	3·19	2·99	93·7	...	...	...	...	0·2	0·3

* β-carotene + neo-β-carotene B.

TABLE II—*concld.*

Number	Variety	Neo-β-carotene B.		α-carotene.		Neo-α-carotene.		Total active pigments in terms of β-carotene.		Vitamin A activity.
		µg./g.	Per cent.	µg./g.	Per cent.	µg./g.	Per cent.	µg./g.	Per cent.	I. U./g.
1	2	8		9		10		11		12
1	Red (Lahore) (a)	*	…	10·2	10·9	0·4	0·4	85·98	91·9	143·3
	Red (Lahore) (b)	*	…	11·4	11·9	0·5	0·5	85·65	89·9	142·8
2	Deep red (Delhi) (a)	20·0	14·3	13·0	9·3	0·8	0·6	116·84	83·7	194·7
	Deep red (Delhi) (b)	16·8	16·2	16·3	15·7	2·0	1·9	81·28	78·5	135·4
3	Red (Delhi)	8·7	10·3	15·7	18·6	1·2	1·4	69·74	82·5	116·2
4	Light red (Delhi)	5·0	7·9	14·5	23·0	1·4	2·2	51·13	81·0	85·2
5	Deep orange (Delhi)	0·3	0·6	26·0	51·9	2·5	5·0	30·95	61·7	51·6
6	Orange (Delhi) (a)	0·9	2·2	13·6	34·0	1·7	4·3	21·66	54·2	36·1
	Orange (Delhi) (b)	1·7	5·4	14·0	44·6	1·3	4·1	13·21	42·0	22·0
7	Pale yellow (Delhi)	…	…	…	…	…	…	0·2	9·1	0·3
8	Light yellow (Delhi) (a)	…	…	…	…	…	…	0·33	7·8	0·55
	Light yellow (Delhi) (b)	0·05	0·7	…	…	…	…	0·33	5·4	0·55
9	Yellow (Delhi) (a)	0·1	1·2	…	…	…	…	1·40	19·0	2·33
	Yellow (Delhi) (b)	0·3	2·4	0·04	0·3	…	…	0·99	11·9	1·65
10	Deep yellow (Delhi)	0·2	1·2	…	…	…	…	2·63	21·0	4·4
11	Pink with a yellow core (Delhi)	…	…	…	…	…	…	2·05	13·0	3·4
12	Violet with a pale yellow core (Delhi) (a)	0·07	1·7	…	…	…	…	0·27	11·2	0·45
	Violet with a pale yellow core (Delhi) (b)	0·05	0·6	…	…	…	…	0·54	13·1	0·90
13	Violet with a yellow core (Delhi)	…	…	…	…	…	…	0·74	9·4	1·2
14	Violet with a pale yellow core (Lahore)	…	…	…	…	…	…	0·2	6·3	0·33

* β-carotene + neo-β-carotene B.

DISCUSSION.

Pigments isolated and identified in the different varieties include xanthophyll. γ-carotene. β-carotene. α-carotene. neo-β-carotene U. neo-β-carotene B and neo-α-carotene. No additional bands appeared. Consequently δ-carotene reported by Strain (*loc. cit.*), and ζ-carotene and lycopene reported by Harper and Zscheile (*loc. cit.*) in some varieties were not identified. γ-carotene was found to be absent from orange and violet varieties although it was detected in appreciable quantities in the deep yellow ones   The pale and light yellow carrots lacked also α-carotene and its isomer neo-α-carotene in addition to γ-carotene.

In the red varieties. β-carotene was found to be the principal pigment varying from 40·4 μg./g. to 99·0 μg./g. constituting 59·8 to 86·0 per cent of the total pigments, while α-carotene was present to the extent of 10·2 μg./g. to 16·3 μg./g. representing 9·3 to 23·0 per cent. The quantity of neo-β-carotene B was also appreciable being 5·0 μg./g. to 20·0 μg./g. or 7·9 to 16·2 per cent of the total. Xanthophyll, γ-carotene, neo-β-carotene U and neo-α-carotene are all present in small proportions up to 3·2 per cent of the total.

In case of orange-coloured carrots. α-carotene was found to be the principal pigment and was present from 13·6 μg./g. to 26·0 μg./g. constituting 34·0 to 51·9 per cent of the total pigments.   The amount of β-carotene in these varieties varied from 4·2 μg./g. to 16·0 μg./g. or 13·3 to 32·5 per cent of the total. Unlike red-coloured carrots which contained xanthophyll only in small quantities, 2·2 to 3·2 per cent, the orange varieties contained this pigment quite in appreciable quantities, 5·1 μg./g. to 9·9 μg./g. or 10·2 to 29·3 per cent of the total pigments. The remaining pigments neo-β-carotene U. neo-β-carotene B and neo-α-carotene were present only in small quantities.

It was interesting to note that in pale yellow to deep yellow and pink varieties, xanthophyll was found to be the principal pigment occurring between 2·0 μg./g. to 13·2 μg./g. or 75·9 to 93·4 per cent of the total pigments. The next pigment of importance was β-carotene though only small quantities are present. 0·2 μg./g. to 2·3 μg./g. or 5·0 to 18·4 per cent of the total. In this group. γ-carotene. α-carotene. and neo-α-carotene were generally not detected except in the case of yellow and deep yellow varieties.

In the case of violet-coloured carrots also. xanthophyll was found to be the principal pigment and was present from 2·1 μg./g. to 7·0 μg./g. or 84·7 to 93·7 per cent of the total.   Like pale and light yellow varieties, γ-carotene, α-carotene and its isomer neo-α-carotene were not detected. The amount of β-carotene was found to be exceedingly small varying between 0·2 μg./g. to 0·7 μg./g. or 6·3 to 11·9 per cent of the total.

The relative vitamin A potency of α- and γ-carotenes is generally recognized to be half that of β-carotene. The potency of neo-β-carotene U has been investigated by a number of investigators. This pigment was reported at first to have no vitamin A activity (Polgar and Zechmeister. *loc. cit.* : Kemmerer and Fraps, 1943 ; Kemmerer, Fudge and Fraps, 1944). Deuel *et al.* (1944) claimed that it was active to the extent of 38 per cent of β-carotene. Later. Kemmerer and Fraps (1945b) carefully examined its vitamin A potency after preparing it from two sources. namely, by iodine catalysis of β-carotene and isolation from a natural source,

alfalfa. They found the vitamin A potency to be 22 per cent in the former and 25 per cent for the latter as compared to β-carotene. The vitamin A potency of neo-β-carotene B was considered to be equal to that of β-carotene by Gillam, el Ridi and Kon (*loc. cit.*) and Mann (1944), though lately it has been found to have only half of the activity of β-carotene (Kemmerer and Fraps, 1943 ; Kemmerer, Fudge and Fraps, 1944). There is only one report in the literature regarding the vitamin A potency of neo-a-carotene (Gillam, el Ridi and Kon, *loc. cit.*). These authors describe it as having 0·7 the activity of β-carotene.

On the basis of the above observations, the biological activity of a-, γ- and neo-β-carotene B have been taken to be half that of β-carotene, that of neo-β-carotene U is 25 per cent and neo-a-carotene as 70 per cent. The total active pigments have been expressed in terms of β-carotene and have been found to vary from 51·1 μg./g. to 116·8 μg./g. or 78·5 to 91·7 per cent of the total pigments in case of red varieties of carrots, 13·2 μg./g. to 30·9 μg./g. or 42·0 to 61·7 per cent in case of orange, 0·2 μg./g. to 2·63 μg./g. or 5·4 to 21·0 per cent in case of pale yellow to deep yellow and pink varieties, and 0·2 μg./g. to 0·74 μg./g. or 6·3 to 13·1 per cent in case of violet varieties.

It will be noticed from the above figures that while the percentage of active pigments as compared to the total approaches 80 to 90 per cent in the case of red and deep red carrots, it is 40 to 60 per cent for orange and only 5 to 20 per cent for light yellow, yellow, pink and violet-coloured varieties. In the latter case especially it will give a totally erroneous idea of the vitamin A activity of this vegetable if it is based on the amount of total carotenoid pigments present as has sometimes been done. This is seen in Table III in which the vitamin A activity calculated on the basis of total hydrocarbon pigments present, and on the basis of individual carotenoids, is shown. The latter is the true vitamin A activity of the product. The first value is 6·3 to 68·2 per cent higher than the true value. The vitamin A activity on the basis of the active pigments is found within a range of 85 I. U. to 195 I. U. per g. for the red varieties, 22·0 I. U. to 51·6 I. U. per g. for the orange, 0·3 I. U. to 4·4 I. U. per g. for pale yellow to deep yellow and pink, and 0·3 I. U. to 1·2 I. U. per g. for the violet.

TABLE III.

Difference in vitamin A activity of carrots by the two methods.

Number.	Variety.		Vitamin A activity calculated on the basis of total hydrocarbon pigments.	Vitamin A activity calculated on the basis of individual active pigments present.	Difference between two values.
1	2		3	4	5
1	Red (Lahore) ...	(a)	152·4	143·3	6·3
		(b)	155·0	142·8	8·5
2	Deep red (Delhi)		225·3	194·7	15·7

TABLE III—*concld.*

Number.	Variety.		Vitamin A activity calculated on the basis of total hydrocarbon pigments.	Vitamin A activity calculated on the basis of individual active pigments present.	Difference between two values.
1	2		3	4	5
3	Red (Delhi)	(a)	167·7	135·4	23·8
		(b)	137·5	116·2	18·3
4	Light red (Delhi) ...		102·2	85·2	19·9
5	Deep orange (Delhi) ...		75·0	51·6	45·3
6	Orange (Delhi) ...	(a)	50·2	36·1	39·1
		(b)	37·0	22·0	68·2
7	Light yellow (Delhi) ...	(a)	0·67	0·55	21·8
		(b)	0·67	0·55	21·8
8	Yellow (Delhi) ...	(a)	2·75	2·33	18·0
		(b)	1·97	1·65	19·4
9	Deep yellow (Delhi) ...		5·03	4·40	14·3
10	Pink with a yellow core (Delhi) ...		4·33	3·40	27·3
11	Violet with a pale yellow core (Delhi).	(a)	0·50	0·45	11·1
		(b)	1·05	0·90	16·6
12	Violet with a yellow core (Delhi) ...		1·38	1·20	15·0

SUMMARY.

The carotenoid· pigments of fourteen varieties of carrots ranging in colour from deep red to orange, yellow and violet have been investigated. Seven different carotenoid pigments, namely, xanthophyll, γ-carotene, β-carotene, α-carotene, neo-β-carotene B, neo-β-carotene U and neo-α-carotene were isolated and identified. The individual carotenoid pigments have been separated by chromatographic analysis, identified and colorimetrically estimated. Detailed data are presented in the paper regarding the amounts of different carotenoid pigments present in different varieties. γ-carotene, α-carotene and neo-α-carotene were found to be absent from certain varieties.

β-carotene is the principal pigment for the red varieties being present to the extent of 60 to 80 per cent. In the orange-coloured varieties α-carotene is the principal pigment being present to the extent of 34 to 52 per cent. whereas

xanthophyll was found to be the principal pigment in light yellow, yellow, pink and violet-coloured varieties. It was found to occur to the extent of 75 to 94 per cent of the total carotenoids present.

Taking into consideration, the biological activities of different carotenoids, the vitamin A potency of each variety has been estimated. It is shown that the only reliable method of determining vitamin A activity of such products is the isolation and determination of individual carotenoids and calculating the vitamin A potency on the basis of their relative activities. The vitamin A potency of the different varieties investigated showed rather a wide range of 0·3 I. U. to 195 I. U. per g.

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STUDIES IN EXPERIMENTAL RICKETS: THE ALKALINE SERUM PHOSPHATASE IN RACHITIC ALBINO RATS.

BY

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IT is well known that in many bone diseases like Paget's disease. osteomyelitis, osteoporosis, osteogenic sarcoma. metastatic bone cancer, etc.. there occurs an increase in the serum alkaline phosphatase (Morris and Peden, 1937). In rickets the increase was noted as early as 1925 by Demuth (Demuth, 1925). Kay (1930) while confirming this observation stated that since the bone as well as the serum enzyme values were increased at the onset of the disease. the serum phosphatase was probably identical with the bone phosphatase. In a later communication, Kay (1932) suggested that the increase in serum alkaline phosphatase in bone diseases was due to a leakage from the bone tissue. According to Bodansky (1934) also the serum phosphatase in bone diseases is of osseous origin. Against this is the fact that the serum alkaline phosphatase increases in many other diseases. e.g. obstructive jaundice (Flood, Gutman and Gutman. 1937). hepatic cirrhosis and hyperthyroidism (Bodansky and Jaffe. 1934*a*). etc.. in which bone is not concerned.

Besides the diseases mentioned above, the dietary constituents may also exert an influence on the serum alkaline phosphatase. Bodansky (*loc. cit.*) observed that in dogs carbohydrate feeding caused an increase in serum phosphatase and no effect was visible when fats were ingested. He suggested that this increase in serum was caused by the excess production of the enzyme in the intestinal mucosa. muscle, etc.. from which it escaped into the serum. This hypothesis receives support from the work of Lundsgaard (1933) and Verzár (1935). Weil and Russell (1940) claim, however, that in albino rats the reduced serum

phosphatase activity caused by prolonged fasting was greatly increased by the ingestion of fats and not by carbohydrates. Freeman and Farmer (1935) have shown that in dogs and rabbits, high protein diets lower the serum phosphatase, while carbohydrates and fats raise it. Choline (Dutra and McKibbin, 1945), ascorbic acid (Gould and Schwachman, 1942) and manganese (Wiese *et al.*, 1939) also have been shown to influence the serum alkaline phosphatase.

Thus, the fact that the serum alkaline phosphatase is increased in bone diseases of diverse ætiological origin and in other diseases in which bone is not involved leads one to suggest that the hitherto accepted view that the bone phosphatase is the source of serum alkaline phosphatase would require further experimental evidence before it can be considered to be conclusively proved.

Another observation reported by Dikshit and Patwardhan (1946) which adds to the doubts regarding the origin of serum alkaline phosphatase is on the time-lag in the fall of the serum phosphatase even after complete healing of the rachitic lesion has taken place. If the hypertrophic cartilage was the source of serum phosphatase in rickets the latter should come down to the normal after it has calcified, degenerated and been replaced by bone ; instead the serum alkaline phosphatase continues at a higher level than the normal for a fairly long time. Why the serum phosphatase level should lag behind the healing of the bone lesion is not satisfactorily explained by the present conception of the source of serum phosphatase.

<center>EXPERIMENTAL.</center>

Albino rats were selected for the experiment since rickets could be induced in these animals in varying degrees of severity within a reasonably short period. Twenty young and healthy albino rats weighing above 35 g. and 31 to 38 days of age were divided into four groups each comprising five rats and fed on diets I to IV. The litter mates were distributed equally in all the groups. The animals were kept individually in cages which were kept in a room where no direct or reflected sunlight could penetrate.

The composition of the rachitogenic diets is given in Table I :—

<center>TABLE I.</center>

<center>*Composition of the various diets with different Ca : P ratios.*</center>

Articles.	Diet I. Ca : P 1·4 : 1	Diet II. Ca : P 5·7 : 1	Diet III. Ca : P 10·2 : 1	Diet IV. Ca : P 15·0 : 1
	g.	g.	g.	g.
Amylum Starch	509	509	509	509
Sesame oil	32·5	32·5	32·5	32·5
Sodium chloride	7·2	7·2	7·2	7·2
Calcium carbonate	9·5	9·5	9·5	9·5
Potassium dihydrogen phosphate	15·4	2·46	0·75	0·31
Egg white	1,000 c.c.	1,000 c.c.	1,000 c.c.	1,000 c.c.

The materials mentioned above were well mixed with water to a thick pasty consistency and dried over a water-bath and in a steam oven, powdered and preserved. The powdered diets were weighed in numbered containers, an aqueous extract of 0·75 g. of yeast per rat per day was added to each and the mixture stirred with sufficient quantity of water to form a thick paste. The food was then steamed for 15 to 20 minutes and on cooling placed in the cages. Unrestricted quantity of tap-water was also kept available in the cages.

Vitamin A in the form of diluted shark-liver oil was administered orally to each rat in biweekly doses equivalent to 25 I. U. per rat per week, but no vitamin D was given.

Bleeding of the animals.—The animals were bled once a week. They were first lightly anæsthetized with ether and 0·3 c.c. to 0·4 c.c. of blood was drawn by heart puncture with a fine dry syringe and needle. Although heart puncture required some experience and skill, it was found more convenient than the method described by Weil and Russell (*loc. cit.*) in which blood was obtained by cutting the tail of the animal. The blood was allowed to clot and the serum separated by centrifuging in a capillary tube, one end of which was sealed. The serum obtained thus was usually clear, only a rare sample showed very slight hæmolysis.

Determination of alkaline serum phosphatase.—The method of King and Delory (1939) using phenyl phosphoric ester as the substrate was used for the determination of alkaline serum phosphatase. In this method the phenol liberated by the action of serum phosphatase upon the substrate phenyl phosphate was determined colorimetrically. Tyrosine solution was used as the standard phenol as described by Binkley, Shanks and Hoagland (1944). The results are expressed as phosphatase units in terms of mg. phenol liberated per 100 c.c. of the serum at 37·5°C. and pH 9 in 30 minutes.

Ash content of femurs.—At the end of the experimental period all the rats were killed after the final bleeding and their femora taken out. The bones were freed from the extraneous soft tissue and extracted first with alcohol for 8 hours followed by ether for 24 hours. They were then dried to constant weight and ashed in platinum or silica crucibles.

Staining of the tibial slices.—The tibiæ were slit open longitudinally and fixed in 10 per cent formaline saline for 24 hours. They were washed repeatedly with distilled water and then kept immersed in 2 per cent silver nitrate for 2 hours in a dark place. At the end of this period the slices were washed free from silver nitrate, with distilled water, and exposed to reflected sunlight for 15 minutes. Only the portion which is calcified is blackened on exposure, the rest remaining white. In this way it was possible to observe the increased width and irregularity of the epiphyseal cartilage in rickets. Camera-lucida sketches of the bones treated in this manner were made.

DISCUSSION.

Ash content.—The ash content of dry fat-free femora of rats obtained at the termination of the experiment is given in Table II. The results indicate that even in rats on diet with Ca : P = 1·4 : 1 the ash content was 46·12 per cent, a figure

below the normal, this being probably due to the absence of vitamin D. The deficient calcification characteristic of rickets was further accentuated in other groups when the disparity between calcium and phosphorus in the diet increased. The average percentages of ash in the other three groups were 31·63, 28·85 and 29·20 respectively.

TABLE II.

The ash content of dry fat-free femora of rats on diets with different Ca : P ratios.

GROUP I. Ca : P 1·4 : 1		GROUP II. Ca : P 5·7 : 1		GROUP III. Ca : P 10·2 : 1		GROUP IV. Ca : P 15·0 : 1	
Rat No.	Ash per cent.	Rat No.	Ash per cent.	Rat No.	Ash per cent.	Rat No.	Ash per cent.
3102	45·81	3103	33·22	3104	26·42	3105	28·17
3106	45·94	3107	33·32	3108	32·72	3109	29·70
3110	46·47	3111	29·03	3112	28·97	3113	32·10*
3114	46·06	3115	31·09	3116	30·13	3117	28·97
3118	46·31	3119	31·51	3120	26·02	3121	29·85
Average	46·12	Average	31·63	Average	28·85	Average	29·20

* Rat died after second week, hence excluded from the average of group IV.

The appearance of the epiphyseal cartilage.—Fig. 1 illustrates the appearance of the epiphyseal plate of cartilage as seen in rats belonging to the four different groups. These camera-lucida drawings are typical of others prepared from the tibiæ of the remaining animals, hence it was considered unnecessary to include all other sketches. The increasing severity of rickets with increasing Ca : P values is clear from these sketches. The epiphyseal cartilages of rats receiving diet with Ca : P = 1·4 were narrow and regular (3102) in this respect not differing from the appearance of a normal epiphyseal cartilage. In the animals of group II (Ca : P = 5·7) the cartilage disc was wider and irregular (3103), while in groups III (Ca : P = 10·2) and IV (Ca : P = 15) the cartilages were still wider and metaphyseal margins more irregular. No appreciable difference could be made out between the last two groups as judged by the appearance of the epiphyseal cartilage or the ash content of the bone.
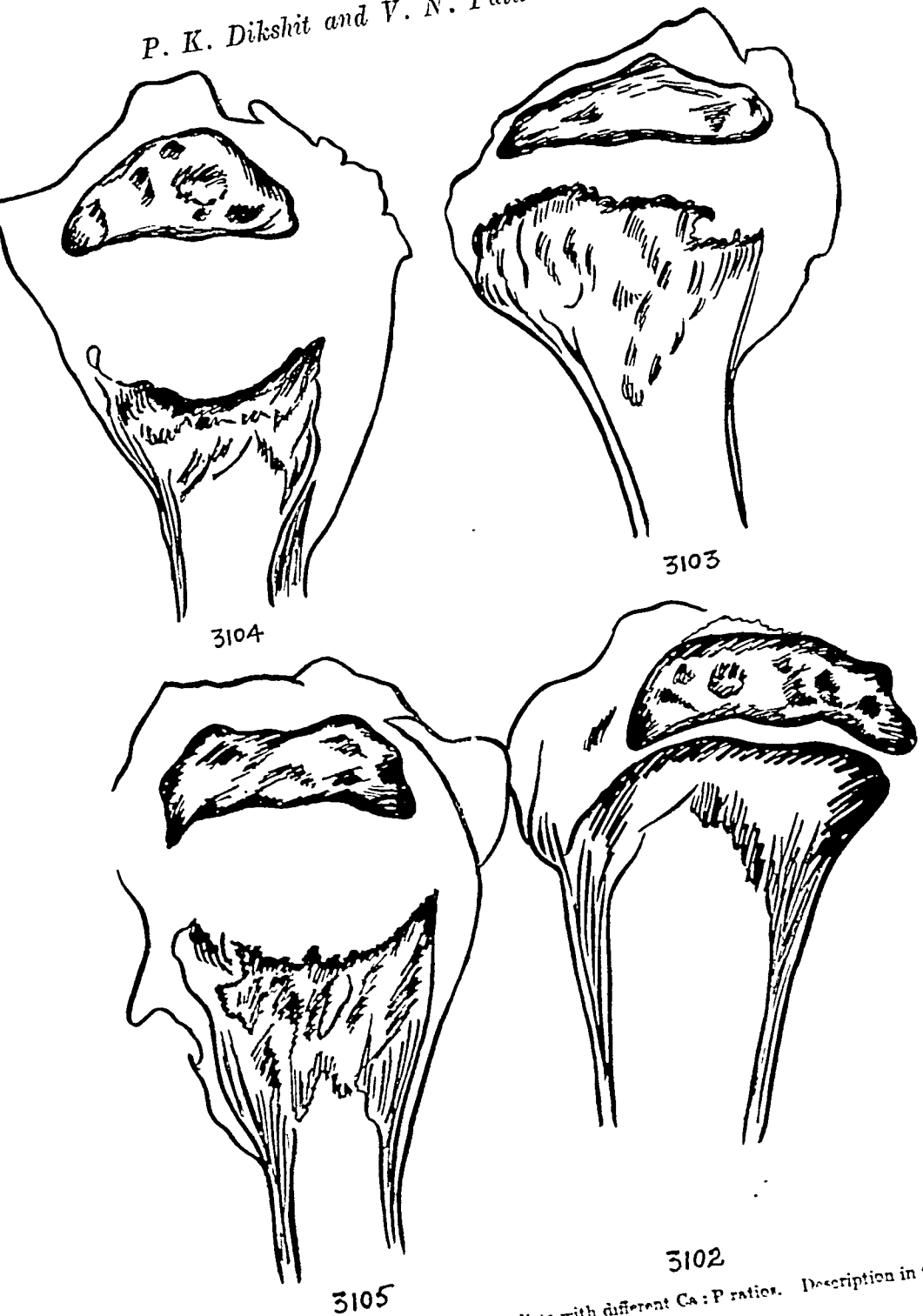
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3104

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3102

Fig. 1. Epiphyses of albino rats on diets with different Ca : P ratios. Description in text.

Serum phosphatase.—The initial serum phosphatase values of the rats varied from animal to animal (Fig. 2). The variation was very large and lay between 175 and 53 units. Neither the litter. age nor the previous dietary history of the animals appeared to have a bearing on these initial figures.
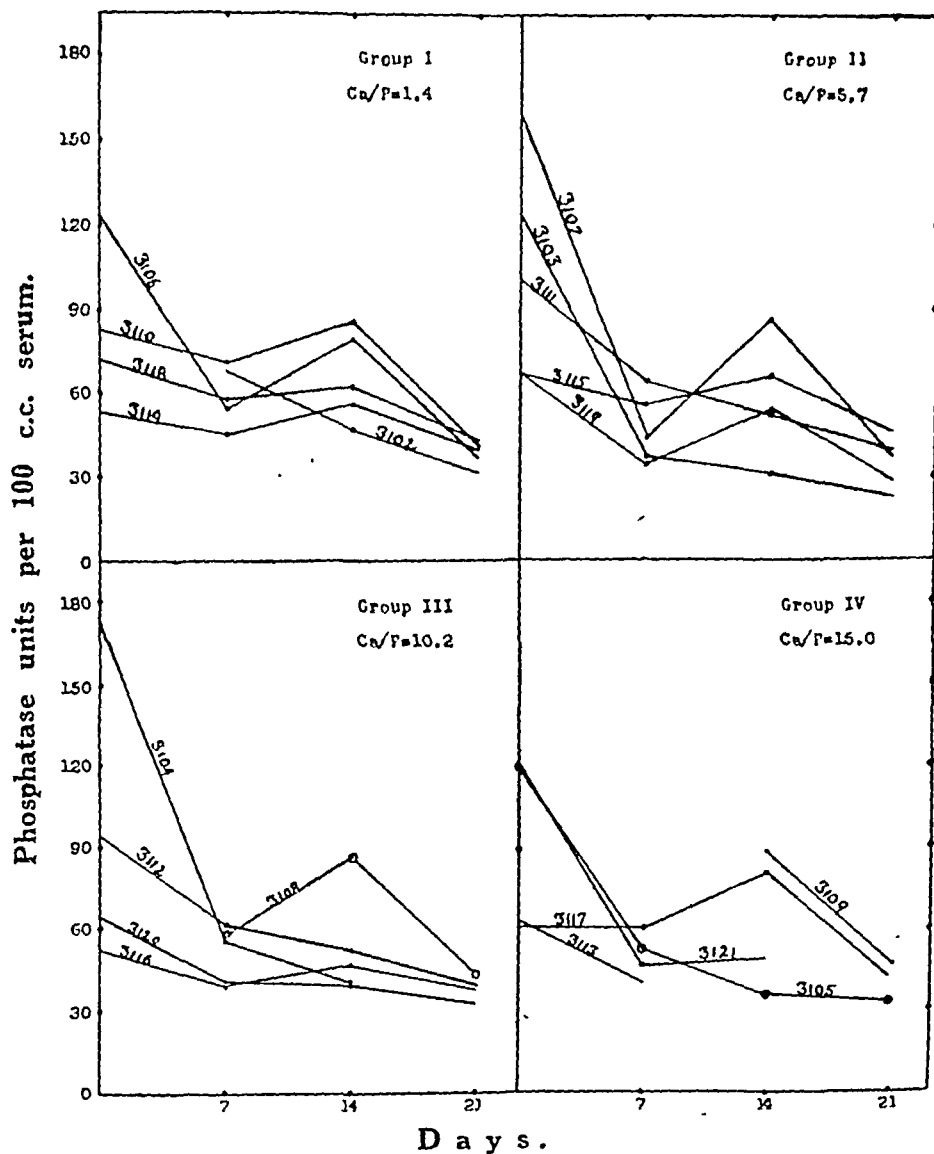

Fig. 2. Influence of dietary Ca : P ratio on the serum alkaline phosphatase.

After the end of the first week on the respective diets the phosphatase showed a fall in all the groups irrespective of the diets. At the end of 2 weeks a slight rise in the phosphatase values was observed in 11 animals. This increase was not confined to any particular group, as is evident from the fact that out of the 11 rats

which showed a rise 4 belonged to the first group and 3 to the second group. In the rest of the animals, however, the fall continued. The end of the third week saw a general fall in the serum phosphatase in all the animals. The final values in various groups ranged between 22 and 48 units (Fig. 2).

It is not possible to explain these results in the light of the generally accepted view that in rickets the serum phosphatase increases considerably. If this view were correct it was only logical to expect that the serum phosphatase would increase in these rachitic rats also. It may, however, be possible that the serum phosphatase value showed a fall on account of the growing age of the rats, but the onset of severe rickets should have caused an increase greater than the fall due to increasing age.

Robison (1923) had observed that the enlarged zone of provisional calcification in the rachitic cartilage of the rat contained active phosphatase. He further found that the long bones of rachitic rats yielded extracts of phosphatase relatively more active than those obtained from comparable bones of normal animals. Hence an increase in bone phosphatase activity in the rachitic animals of the present series is to be expected. As no determination of the bone phosphatase was made in the present investigation, no definite statement to this effect can be made. Considering that marked rickets was present in the animals of groups II to IV showing an increase in the osteoid tissue and the hypertrophic cartilage which are the characteristic features of rickets one can reasonably assume that there must have been an increase in bone phosphatase in these rachitic animals. In such a case a concomitant increase in the serum phosphatase activity should be expected. Since the serum phosphatase value was continually decreasing the suggestion, however, seems to be not altogether unjustified that the source of increased serum phosphatase in rickets observed by other workers could possibly have been other than the rachitic bone.

It must be mentioned here that Freeman and McLean (1941) who produced a very severe rachitic condition in dogs could observe only a threefold increase in serum phosphatase activity. This is in strong contrast to the observations of Kay (1932) and Bodansky and Jaffe (1934b) who observed a twenty-fold increase in serum phosphatase activity in rachitic children. Even then the latter could not find a correlation between the intensity of rickets and serum phosphatase values.

The fact that no positive controls with vitamin D were kept in the present experiment will require some explanation. Dikshit and Patwardhan (*loc. cit.*) had observed that the serum alkaline phosphatase activity in dogs started increasing from the very beginning of vitamin D deficiency, but that it did not keep pace with the increasing severity of rickets as judged by the x-ray plates. It was with a view to testing the relationship between serum phosphatase activity and the severity of the rachitic lesion that these experiments on albino rats were undertaken. It will be clear, therefore, that for such a purpose the absence of positive controls does not affect the conclusions reached.

The onset of the deficiency of vitamin D as already stated has been known to cause an increase in serum phosphatase which is still generally considered to be of osseous origin. The findings described in this paper and the anomalies mentioned

earlier do not give support to this hypothesis; on the contrary they throw considerable doubt on the validity of the views of Kay (1932) and Bodansky (*loc. cit.*).

SUMMARY.

1. Experiments were carried out on healthy young albino rats to determine the correlation between the intensity of rickets and the serum alkaline phosphatase activity. The animals were divided into four groups and fed for 3 weeks on diets having Ca : P ratio 1·4, 5·7, 10·2 and 15·0 to 1 respectively.

2. Each animal was bled once a week for 3 weeks and the alkaline phosphatase was determined in the serum.

The initial serum alkaline phosphatase values were quite high, but at the end of the first week on rachitogenic diets, a considerable decrease in the values was noticed in all the rats. At the end of the second week, however, a slight rise in the phosphatase value occurred in more than half the number of animals; but at the conclusion of the experiment, the phosphatase activity again markedly decreased in all the animals.

3. · At the end of 3 weeks all animals were killed; the tibiæ slit longitudinally and stained by von Kossa's method were examined.

It was observed that the epiphyseal cartilages of the rats receiving diet with Ca : P ratio of 1·4 were normal in appearance, but with an increase in the dietary Ca : P ratio the epiphyseal cartilage disc became considerably wider and more irregular. The epiphyses of rats on the diet with a Ca : P ratio of 5·7 were appreciably wide and irregular. With the Ca : P ratio at 10·2, the cartilage had still further widened. No difference in the width of the cartilages of rats on diets with Ca : P = 10·2 and 15·0 was observed.

4. The average values of ash in the dry and fat-free femurs of the four groups were 46·12, 31·63, 28·85 and 29·20 per cent respectively.

5. As no increase in the serum alkaline phosphatase was observed in spite of a severe rachitic condition as shown by (a) the increased epiphyseal cartilage width and (b) decreased bone-ash content, it is suggested that in rickets the serum alkaline phosphatase may not necessarily originate from the bone. The fact that other observers have found lack of correlation between serum alkaline phosphatase and severity of rickets in children and in dogs lends support to this view.

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THE EFFECT OF PREGNANCY ON THE INCIDENCE OF DENTAL CARIES IN INDIAN WOMEN.

BY

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PERUSAL of the available literature and research data relative to the incidence of dental caries during pregnancy only serves to demonstrate the wide divergence of views regarding the popular idea that pregnancy increases the susceptibility to caries. There is an abundance of lay and medical testimony of a more or less empiric nature to support this hypothesis. Price (1932) states. ' a study of the history of peoples as revealed by the idioms of their language has disclosed that practically every race with a language has a phrase equivalent to " a tooth for every child " notwithstanding these languages may have had their origin in the dim and distant past '. It would seem that this can be accounted for only on the basis of the recognition of a clinical fact and ' an expression of the well-known tendency to de-mineralization of the maternal tissues. the bones and teeth especially ' (Day, 1944).

In more recent times added support has been given to this view by the unjustified claims that nutritional factors determine the incidence of dental caries. Investigations carried out during the past few years in the Kangra valley and Hissar district in India lead to the conclusion that the nutritional deficiency and vitamin hypotheses are untenable (Taylor and Day, 1939, 1940; Day, *loc. cit.*).

Local as well as general systemic factors have from time to time been suggested as the possible cause of this apparent deterioration of the teeth during the gestation period—nausea and vomiting or ' morning sickness ' with its eruotationa af +h

acid contents of the stomach into the oral cavity, so changing the normal alkalinity of the oral secretions; changes and perversions in appetite; ptyalism and change in the normal alkalinity of the saliva even when regurgitation to the stomach contents does not occur. This increasing acidity of the saliva in combination with the defective mouth hygiene has been frequently suggested as an important contributing local factor in the alleged destructive effects of pregnancy on the teeth.

Although pregnancy and lactation have come to be considered as normal and physiological states and not in any sense pathological as has been held in the past, yet these functions do constitute a period of increased stress on the part of the maternal organism, as the mother is faced with the double responsibility of supplying proper amounts of nutrients necessary for the offspring as well as for her own body-metabolism. The fact that the teeth are the most highly calcified structures in the human body has focused attention on the calcium metabolism of the mother during gestation and the possibility of calcium starvation during this period causing withdrawal of calcium from the teeth as well as from the bones. Bogert and Plass (1932) found that the calcium content of the foetal serum is consistently higher at the time of birth than in the mother and exceeds the normal calcium range of non-pregnant women. There is much evidence to support the view that malnourished mothers give birth to seemingly well-nourished and fully-developed children, indicating that the foetus has the ability to extract substances from the mother's circulation to meet the needs of its own growth, and when these nutritional elements do not exist in the maternal serum in adequate amounts the mother's nutritive reserve may be drawn on to meet the needs of the child.

Despite the contrary view held by some investigators it can be stated that the theory of the withdrawal of calcium from the teeth during pregnancy in any circumstances has not been adequately established. Most of the biological studies (Karlstrom, 1924: Toverud. 1927; Rosebury and Foley. 1934) relating to pregnancy and dental caries have been carried out with rats' incisor teeth and should be largely discounted in view of the fact that analytical comparisons cannot reasonably be made as the incisors of these animals are of continuous growth from a persistent pulp. Dragiff and Karshan (1943) analysed for percentage of ash the root dentine of 31 teeth of pregnant women and 21 teeth of non-pregnant women. They found no difference between the two groups and concluded that there was no basis for the view that minerals are withdrawn from the teeth of humans during pregnancy.

The results of clinical investigations are so much at variance that they provide scant proof on a strictly scientific basis of a greater deterioration in the teeth of pregnant women.

The findings of Ballantyne (1919). that 98 per cent of pregnant women show carious teeth. have little significance in view of a similar high caries incidence frequently reported amongst non-pregnant women, males and children. Bodecker (1930) reports the frequent finding of an increase of dental caries during pregnancy and suggests that the cause may be a reduction of mineral salts in the teeth which would increase their permeability. Ziskin (1926) examined 599 pregnant women during the last months of pregnancy, and 205 non-pregnant women. He found a

lower incidence of decayed teeth in the pregnant group. In a subsequent study Ziskin and Hotelling (1937) produced evidence to show that pregnancy not only does not increase tooth decay but that factors operating during pregnancy actually prevent caries to a significant degree. Similar conclusions were reached by Starobinsky (1929) who investigated the dental caries incidence in 150 non-pregnant women and 216 pregnant women at the ninth month of pregnancy. Gomperts (1926) draws similar conclusions from his own investigations. On the other hand Gerson, quoted by Weintraub (1932), made observations of 50 pregnant women between the second and fourth month, and 50 non-pregnant women of the same age and social status. The whole series of 100 were examined six months later. His results based on teeth lost and caries incidence during the period of investigation strongly suggested a positive correlation between pregnancy and dental caries. Except for one reference quoted by Weintraub (*loc. cit.*) to the investigations of Lintz, who reported a positive correlation, the available literature would seem to indicate that no attempt has been made to correlate the incidence of dental caries and the number of pregnancies in the same age group. If pregnancy has an effect on dental caries incidence, whether the cause be local or systemic, it would seem safe to assume that the incidence of dental caries will be in direct proportion to the number of pregnancies in groups of the same age and social status.

The present study was designed to determine whether any relationship does in fact exist between the incidence of dental caries and the number of pregnancies in various age groups.

METHOD OF INVESTIGATION.

This was the same as that followed in earlier surveys (Shourie. 1941, 1942, 1946). A detailed dental inspection, in which all the available tooth surfaces in the mouth were examined using a dental mirror and probe, was made in the case of each individual. The total number of teeth and the number of *deciduous* and permanent teeth (when present) were recorded. All extracted teeth were recorded as carious, except when known to have been lost as the result of trauma. paradontal disease or extracted for some other reason. In recording the extent of caries the classification of Day and Sedwick (1934) was employed, which is as follows :—

1. Initial caries including softened or discoloured pits and fissures giving lodgment to fine explorers.

2. Freely accessible approximate cavities and small open cavities involving less than one-quarter of the tooth.

3. More extensive caries involving more than one-fourth and less than two-thirds of the crown.

4. Caries involving from two-thirds to complete destruction of the crown.

The average caries figure ... $\dfrac{\text{Total caries figures}}{\text{Number of teeth examined}}$.

The total caries figures are calculated by multiplying the number of teeth under each of the heads mentioned above by the corresponding number, i.e. 1, 2, 3 or 4. as the case may be. One hundred and eighty-two women were examined.

These were in-patients in the Lady Willingdon Hospital, Lahore, and those attending the out-patients' department of the same institution.

In each case information was also collected regarding the age and the number of full-term deliveries. For analysis of data different individuals were divided into various age groups. Persons in each age group were further grouped with regard to the number of pregnancies. Incidence of dental caries was compared in the different pregnancy groups.

Table I shows the incidence of dental caries in relation to the number of pregnancies in all the age groups, while Tables II. III and IV give the caries incidence in the various age groups and the relationship of dental caries with regard to the number of pregnancies in each age group.

In each table comparisons have been made with regard to :—

(1) The percentage of persons free of caries.

(2) The percentage of carious teeth—

    (*a*) total number of teeth.

    (*b*) lower left molars only. This has been done to meet with the statistical objections that different teeth in the mouth cannot be taken as similar entities as their forms. functions, and eruption times are different.

DISCUSSION AND RESULTS.

Table I indicates that there is no significant difference in dental caries incidence in subgroups arranged according to the number of pregnancies. Had there been any effects of pregnancy on the dental caries process, the incidence of caries would have been expected to increase in proportion to the number of pregnancies.

The results were statistically analysed and were not found to be significant.

To eliminate the effect of age in Table I, persons studied were divided into the following age groups :—

    A. From 15 to 20 years of age (Table II).

    B. From 21 to 30 years of age (Table III).

    C. From 31 to 40 years of age (Table IV).

Data collected for each age group were further submitted to statistical analysis regarding the relationship of dental caries to the number of pregnancies, and the differences were again not found to be statistically significant.

Thus, the evidence produced in this investigation does not support the popular concept of a relationship between pregnancy and dental caries incidence.

SUMMARY.

One hundred and eighty-two women were examined for dental caries. Information was also collected regarding the number of pregnancies in each case.

Analysis of the available data does not support the popular concept of a relationship between pregnancy and dental caries incidence.

TABLE I.

Incidence of dental caries in all age groups.

Number of pregnancies.	PERSONS.		TEETH.				
			TOTAL.			LOWER LEFT 1ST MOLAR.	
	Number of persons examined.	Per cent caries free.	Number.	Per cent carious.	Average caries figure.	Number.	Per cent carious.
0	26	50·0	788	3·9	0·08	26	11·5
1	34	41·2	924	5·7	0·12	34	14·7
2	28	46·4	851	4·8	0·10	28	25·0
3	20	55·0	610	4·0	0·07	20	25·0
4	23	52·1	712	6·4	0·14	23	30·4
5	16	50·0	503	9·4	0·26	16	25·0
6	9	22·2	285	4·8	0·10	9	11·1
7	9	55·5	281	4·0	0·11	9	11·1
8	4	50·0	125	2·4	0·04	4	0·0
9	3	100·0	96	0·0	0·00	3	0·0
10	5	60·0	160	2·8	0·05	5	20·0
11	1	0·0	32	3·1	0·10	1	0·0
12	1	100·0	32	0·0	0·00	1	0·0
13	2	0·0	64	3·2	0·10	2	0·0
14	1	0·0	32	3·1	0·10	1	0·0

TABLE II.

Incidence of dental caries in age group 15 to 20 years.

Number of pregnancies.	Average age in years.	PERSONS.		TEETH.					
				TOTAL.			LOWER LEFT 1ST MOLAR.		
		Number of persons examined.	Per cent caries free.	Number.	Per cent carious.	Average caries figure.	Number.	Per cent carious.	
0	18·0	12	33·3	353	3·3	0·04	12	8·3	
1	18·5	17	52·9	403	3·2	0·04	17	11·7	
2	18·4	5	60·0	146	3·4	0·06	5	20·0	
3	20·0	4	50·0	124	7·2	0·09	4	25·0	
4	19·0	41	100·0	30	...	...	1	0·0	
5	20·0	1	0·0	28	...	...	1	0·0	

TABLE III.

Incidence of dental caries in age group 21 to 30 years.

Number of pregnancies.	Average age in years.	PERSONS.		TEETH.					
				TOTAL.			LOWER LEFT 1ST MOLAR.		
		Number of persons examined.	Per cent caries free.	Number.	Per cent carious.	Average caries figure.	Number.	Per cent carious.	
0	24·8	8	62·5	248	2·7	0·07	8	12·5	
1	25·0	16	31·2	290	8·1	0·19	16	18·7	
2	24·0	21	47·6	641	5·1	0·10	21	28·5	
3	25·2	18	50·0	390	2·5	0·07	13	15·4	
4	26·1	14	64·3	437	5·5	0·12	14	21·4	
5	27·5	7	57·1	229	4·0	0·10	7	28·4	
6	28·1	6	33·3	192	6·7	0·13	6	16·6	
7	29·1	5	100·0	155	0·7	0·01	5	0·0	
8	27·0	2	50·0	61	1·5	0·01	2	0·0	
Over	25·5	2	0·0	64	3·0	0·03	2	50·0	

TABLE IV.

Incidence of dental caries in age group 31 to 40 years.

Number of pregnancies.	Average age in years.	PERSONS.		TEETH.				
				TOTAL.			LOWER LEFT 1ST MOLAR.	
		Number of persons examined.	Per cent caries free.	Number.	Per cent carious.	Average caries figure.	Number.	Per cent carious.
0	31·8	5	60·0	153	7·1	0·18	5	20·0
1	35·0	1	0·0	31	0·0	0·00	1	0·0
2	36·5	2	0·0	64	4·7	0·19	2	0·0
3	32·5	2	0·0	64	9·3	0·09	2	100·0
4	31·0	8	37·5	215	8·9	0·20	8	50·0
5	29·2	7	42·8	224	11·0	0·44	7	28·5
6	35·0	2	0·0	61	1·6	0·01	2	0·0
7	37·5	4	0·0	126	10·3	0·22	4	25·0
8	31·0	1	100·0	32	0·0	0·00	1	0·0
9	36·0	3	100·0	84	0·0	0·00	3	0·0
10	35·0	2	100·0	64	0·0	0·00	2	0·0
11	38·0	1	100·0	32	3·1	0·10	1	0·0
12	35·0	1	100·0	32	0·0	0·00	1	0·0
13	40·0	1	0·0	32	0·0	0·00	1	0·0
14	40·0	1	0·0	32	0·0	0·00	1	0·0

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USE OF TOLU BALSAM IN RAMON'S FLOCCULATION REACTION.

BY

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AND

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RAMON (1922) introduced the flocculation test for determining the potency of diphtheria antitoxins. In this *in vitro* method, a constant volume of toxin is brought in contact with graded amounts of antitoxin. The first tube to show flocculation, when observed in a bath at constant temperature, was considered to be the tube where equivalent quantities of toxin and antitoxin were neutralized. The method would have replaced Ehrlich's method, but for certain irregularities which were noted later. Glenny, Pope and Waddington (1925) referred to the discrepancy between the *in vitro* and *in vivo* methods. The difference between the results in the two methods definitely indicates that the first tube to flocculate in Ramon's series is not the tube which represents a truly neutral mixture of toxin and antitoxin. Glenny and Barr (1932) believed this might be due to the lack of firmness in the union that takes place between the antigen and antibody. In fact, subsequent observers, including Taylor, Adair and Adair (1934). Heidelberger and Kendall (1935) and others have noted that invariably the tube containing slight excess of toxin or antitoxin is the first tube to flocculate in the 'β' procedure.

A fresh study of the toxin-antitoxin flocculation reaction was initiated with a view to introducing into the toxin-antitoxin system certain agents which might facilitate the binding of the antigen and antibody. Non-specific substances have

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been used in the past for bringing about or accentuating immunological reactions. Thus. Dold (1921) tried to introduce certain agents. such as kaolin, starch, inulin and agar with the intention of facilitating the agglutination reaction. Meinicke (1922) introduced tolu balsam in the precipitin reaction for syphilis with the same object. The introduction of cholesterol by Sachs (1917) also helped to improve the syphilitic precipitin test.

Various reagents. such as cholesterol. gum resin (from areca-nut), iron albuminate. and tolu balsam were tried. It was found that only tolu balsam in the form of a stabilized colloidal emulsion suited our purpose.

EXPERIMENTAL.

Tolu balsam is thoroughly washed three or four times in hot distilled water. It is then dried, carefully weighed, and dissolved to form a 3 per cent solution in absolute alcohol.

0·4 c.c. of the 3 per cent alcoholic solution of tolu balsam is added to 0·2 c.c. of cardiolipin (Wassermann antigen without cholesterol). The cardiolipin helps in the stabilization of the emulsion.

One c.c. of distilled water is now added to the tube containing the above mixture and mixed thoroughly by transferring the contents from one tube to another several times. This gives a homogeneous milky-white, stable emulsion. It is allowed to stand for about 5 minutes at room temperature before use.

Two series of flocculation tubes are put up. In both series, graded quantities of antitoxin are put in tubes containing 1·0 c.c. of toxin. Into one set 0·1 c.c. of tolu-balsam cardiolipin emulsion is added to each tube and the final volume is made up to 2·1 c.c. with physiological saline.

In the other series, which serves as ' control ', only physiological saline is added to make up the final volume to 2·1 c.c.

In each tube, the contents are thoroughly mixed by inverting gently two or three times. Tolu balsam gives a slight opalescence to the tubes of this series. Both the sets are observed in a water bath at 52°C. with adequate arrangements for illumination. The first tube to flocculate and time of flocculation are noted for each series.

It has been observed that the detection of the first tube to flocculate in the series with tolu balsam is definitely facilitated by the larger size of floccules, and the clearing up of the opalescence with the commencement of the flocculation. The flocculating tube stands out in marked contrast to tubes on either side. The fine floccules of the Ramon's series makes it difficult to detect the first tube to flocculate, and unless the eye is specially trained it is easy to miss the tube. It was also noted that the first tube to flocculate was not the same in both the series.

In all. 3 samples of horse serum after a full course of immunization. and 6 samples of refined diphtheria antitoxin were put up for flocculation. The 9

samples were also put up for *in vivo* titration according to Römer's technique. The results are recorded in Table I :—

TABLE I.

Sample.	I.U./c.c. determined by Ramon's method (approximate value).	Kf.	I.U./c.c. determined by modified method (approximate value).	Kf.	I.U./c.c. determined by Römer's method (approximate value).
HORSE SERUM :					
222 ...	430	25′	500	23′	500
223 ...	500	20′	600	20′	625
226 ...	500	25′	600	20′	625
FINAL REFINED :					
201 ...	1,500	30′	2,000	20′	1,900
202 ...	900	60′	1,200	40′	1,250
203 ...	1,300	20′	1,200	15′	Slightly less than 1,200
204 ...	1,300	40′	1,500	15′	1,450
205 ...	1,300	20′	1,500	13′	1,500
206 ...	1,300	50′	1,500	30′	1,600

In the next series of experiments. 6 samples of diphtheria toxin, prepared at the Haffkine Institute, were tested against a sample of antitoxin whose potency had been carefully determined. The sets were arranged as in the previous experiment. Both sets were observed in a bath at 52°C. with suitable arrangement for illumination. As in our experience with the previous experiments. the detection of flocculation in the series with tolu balsam was a matter of ease. The first tube to flocculate was not the same in both the series. as was evident also in the previous series of experiments. After determining the ' Lf ' by the flocculation method the

samples were tested according to Römer's technique on rabbits. The results are recorded in Table II :—

TABLE II.

Toxin sample.	Ramon's method.		Modified method.		Römer's method I.U./c.c.
	Lf I.U./c.c.	Kf.	Lf I.U./c.c.	Kf.	
251　...	27	30'	31·5	23'	30
236　...	36	19'	40·5	17'	40
252　...	90	8'	80	5'	80
256　...	31·5	28'	27	28'	25
254　...	25	40'	20	35'	20
245　...	13·5	120'	13·5	60'	12·5

From Tables I and II it will be seen that the time of flocculation is definitely hastened in the series with tolu balsam. Tolu balsam, therefore, improves the flocculation reaction (Ramon's) by :—

(a)　Showing a better correlation between the *in vitro* and *in vivo* values.

(b)　Facilitating detection of the first tube to flocculate.

(c)　Shortening the time of flocculation.

It may be noted here that in order further to improve the detection of flocculation, we introduced certain colouring agents. Different dyes were tried. It was finally found that India ink, in the proportion given below, helped to some extent to bring out the floccules in contrast with the surrounding medium.

0·05 c.c. of India ink is added to 2·0 c.c. of physiological saline. This is immediately added in 0·1 c.c. quantities to the tubes containing the toxin-antitoxin tolu-balsam mixture.

DISCUSSION.

In the flocculation reaction, the toxin-antitoxin molecules unite in a lattice and the complex appears as floccules. The union does not take place in an irregular and haphazard manner, but obeys certain laws of proportion. In both the ' a ' and ' β ' procedure, it takes place in certain optimal proportions. Theoretically the first tube to show flocculation should be the tube where equivalent quantities of toxin and antitoxin unite, leaving neither antigen nor antibody in the supernatant. But Taylor *et al.* (*loc. cit.*), Heidelberger and Kendall (*loc. cit.*)

and others have shown that this does not happen in practice. The supernatant invariably contains either an excess of toxin or antitoxin. Evidently this discrepancy between theory and practice is due to some factors influencing the mechanical binding of antigen and antibody. Tolu balsam, as is evident from the experiments, plays a definite rôle in the process of flocculation. The agreement between the results obtained by *in vitro* and *in vivo* methods by the introduction of tolu balsam suggests that the reagent is definitely playing an intermediate rôle in helping better binding of the toxin and antitoxin molecules. This helps the process of neutralization of equivalent quantities of toxin and antitoxin by each other. Just as cholesterol in the Wassermann reaction, as Eagle (1937) believes, serves as a centre of adsorption, colloidal tolu-balsam particles may be serving as an intermediary which facilitates mutual combination between toxin and antitoxin molecules.

SUMMARY.

1. Ramon's flocculation test is modified by the addition of emulsion of tolu balsam stabilized with cardiolipin.

2. The results obtained by the modified flocculation test agreed with those obtained by Römer's technique.

3. The detection of the earliest flocculating mixture is facilitated by the introduction of small amounts of tolu balsam in the system.

4. The time of flocculation is also considerably shortened by the introduction of tolu balsam into the system.

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NOTICE.

The following has been received for announcement.

—*Editor, I.J.M.R.*

31st January, 1947.

THE FOURTH INTERNATIONAL CANCER RESEARCH CONGRESS.

THE Fourth International Cancer Research Congress will be held in St. Louis, Missouri, U.S.A., during September 2 to 7, 1947. The Union Internationale Contre le Cancer having accepted the invitation of the American Association for Cancer Research, the Congress will be held under the joint auspices of these two organizations, with Dr. E. V. Cowdry, Professor of Anatomy, Washington University School of Medicine and Director of Research of the Barnard Free Skin and Cancer Hospital, serving as President of the Congress.

NOTE ON THE RELATIONSHIP OF THE SO-CALLED OGAWA AND INABA TYPES OF *V. CHOLERÆ*.

BY

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As several dissertations on the antigenic structure and serology of *V. cholera* appeared shortly before and during the course of the second World War (e.g. Heiberg. 1935 ; Vassiliadis. 1937 ; Linton. 1940 ; Taylor, 1941) and as Burrows, Mather, McGann and Wagner (1946) have recently summarized, with ample references and in a journal readily accessible, existing knowledge of the subject, there is need only. in now discussing the stability of the Inaba and Ogawa subtypes of this organism, to recall the few published observations and opinions immediately relevant to the issue.

First in sequence and importance in this connection is the work of Kabeshima (1918) who, in reporting the serological difference he had found to exist between a Japanese epidemic vibrio strain 'J ' and a strain 'F' from sporadic cholera in Formosa, stated that he had observed mutation from one serological type to the other. By repeatedly passaging strain 'F' in broth containing the homologous anti-'F' serum he led it to adopt the serological behaviour of strain 'J ' and. though in experiments along similar lines he failed to induce strain 'J' to assume the serological character of 'F'—obtaining at the trial what we can now recognize as a rough variant. spontaneously agglutinable—he claimed to have observed this type of change when an El Tor vibrio. originally adhering to the 'J' pattern. was exposed during growth to the action of 'J' antiserum. Kabeshima further stated that when he inoculated strain 'F' into the gall-bladders of 11 rabbits.

he obtained from these after 37 days '*une souche comparable à la souche J* ', but that when strain ' J ' was similarly inoculated into 7 rabbits '*2 ont fourni une souche qui ne se confond avec la souche F qu'au point de vue de l'immunité* '—an exposition which seems on both scores a little cryptic.

Since the strains ' J ' and ' F ' were the respective prototypes of the Original and Variant (Varié) types of later Japanese, and of the Inaba and Ogawa types of later British authors, the observations of Kabeshima amounted to a demonstration of mutation from the Ogawa to the Inaba type with a rather less convincing suggestion of possible change in the reverse direction.

Kabeshima's statement as to the lability of his serological types under the action of their homologous antisera has been widely cited and as often brushed aside, partly perhaps because of its ungarnished brevity, partly certainly, as the comments of Heiberg (*loc. cit.*), Vassiliadis (*loc. cit.*), and Burrows *et al.* (*loc. cit.*) show, because it has been assumed that, being unaware of the niceties of ' O ' and ' H ' analysis and the subtleties of ' R ' variation, he was not in a position to assess correctly the significance of his observations.

Such an estimate of Kabeshima's work was not likely to be modified by perusal of the communication of Aoki and Oshiro (1934), who pretended to see in the Original and Variant types of Nobechi specific and non-specific phases comparable with those of the Salmonella group. The main evidence of transmutation which these workers were able to adduce lay in their finding that certain cultures, received from other laboratories and labelled as strains of the Original and Intermediate (Hikojima) types, agreed on examination with their so-called non-specific phase. They put forward the thesis that during laboratory cultivation all cultures of *V. choleræ* tend to lapse into this serological form.

Such has not, however, been the general experience—nor our own. Nishimura (1938), whom we quote from the summary of Burrows *et al.*, reported that Nobechi's type-specific strains were still specific 15 years after isolation and those cultures in our possession which purport to represent the original Inaba and Ogawa type strains have, with many other classified cultures since received, remained similarly steadfast.

Prior to the recent World War one of us made some rather desultory attempts to induce modification of the specific serology of the cholera vibrio by cultivating it, with and without addition of complement, in media containing the homologous antiserum—a method of treatment he had found useful in the isolation of rough races from smooth vibrio cultures (White, 1935). These experiments made with ordinary ' O ' or ' H ' + ' O ' antisera and focused mainly on the Inaba type led to no observation of new interest : roughening was the only form of serological change detected.

It, however, seemed possible to us when re-considering the question of the lability of the serological types of the organism that, as a stimulus to change or as a selective aid in observing change naturally occurring, the action of strictly type-specific sera from which the common group component had been removed might succeed where the broader action of the natural sera hitherto employed had failed.

This has proved, at least in part, to be the case and we are satisfied on the evidence detailed below that the Inaba type may consistently be isolated from cultures of the Ogawa type of *V. choleræ*.

MATERIALS.

The strains of V. choleræ *and* V. El Tor *examined.*—For the purpose of our inquiry the following strains were selected from the collection of cultures maintained at the Central Research Institute, Kasauli :—

V. choleræ.

Ogawa type strains.—

> M.L. 15 and M.L. 66—isolated respectively in 1943 at the District Laboratory, Calcutta, and in 1945 at No. 9 Field Laboratory, S.E.A.C.
>
> 358-203A, 368-213B, 534-380A, 536-382C, 551-397C, 843-694B—all isolated in the spring or summer of 1946 at the School of Tropical Medicine, Calcutta.
>
> ' 2586 '—old laboratory strain : no records available.
>
> ' X '—a strain previously received from the collection by one of us (D. L. S.) to serve as an Ogawa type culture in serological and chemical studies proceeding : its serial number having been lost, it may possibly correspond with one of the strains listed above.

Inaba type strains.—

> Inaba (Tokyo)—presumably the original Inaba type strain.
>
> 191A and 194A—strains of unknown origin ; long maintained in the collection.
>
> 596B—isolated in 1943 at the School of Tropical Medicine. Calcutta.
>
> Khulna I—isolated about 1940 by the Bengal Cholera Field Inquiry.
>
> 370-215A. 389-234B, 709-556C. 726-575A, 794-645A. 916-769B. 918-771A, 953-806C—all isolated between the spring and summer of 1946 at the School of Tropical Medicine, Calcutta.

El Tor vibrios.

Ogawa type strains.—

> 34-D 11, 34-D 24—strains isolated at El Tor by Dr. Doorenbos in 1934.
>
> El Tor Doorenbos—an unnumbered strain previously received by D. L. S. from the Central Research Institute collection. It may correspond with one of the El Tor strains noted above.

Inaba type strains.—

> 34-D 13. 34-D 25. 34-D 26—strains isolated at El Tor by Dr. Doorenbos in 1934.

The antisera used in the experiments.—Through the bulk of the work we have used mono-specific agglutinating antisera for the Ogawa and Inaba types of

V. choleræ manufactured at the Central Research Institute for routine purposes. These sera, stored in sealed phials and preserved with phenol, were prepared, we are told, by treating the respective natural ' O ' antisera raised in rabbits with heat-killed dried and pulverized cultures of the heterologous type to remove the common agglutinins and were clarified by centrifugalization. We have been at pains to satisfy ourselves as to their sterility. Indeed it was found that even when diluted 1 in 2 with broth and incubated at 37°C. they were rapidly lethal to a heavy vibrio inoculum by virtue of their phenol content. Two samples of C.R.I. mono-specific Inaba type serum were available to us : one dated 4th June, 1946, and declaring on the label the titres Inaba 1 : 500, Ogawa 1 : 50 ; the other dated 28th November, 1946, claiming an Inaba titre of 1 : 250 (this serum had no action on our Ogawa strains at a dilution of 1 in 20). Only one sample of C.R.I. mono-specific Ogawa agglutinating serum could be supplied to us : this, dated 21st August, 1946, was labelled Ogawa titre 1 : 500. We have found it without action on the typical Inaba type strains in our possession.

During the course of our study, with the object of excluding phenol from our media, we prepared an additional mono-specific Ogawa type serum by exhausting a natural serum with a washed and heat-killed (boiling water-bath) culture of the Inaba type, clarifying it by centrifugalization and finally sterilizing it by filtration through an Elford Gradacol membrane (average pore size 0·66μ).

This had a specific titre of 1 : 2,000 and had no action on the Inaba type as represented by the original Inaba culture and similar strains.

METHODS.

The impossibility of handling and comparing adequately, during the relatively short period of our collaboration, large numbers of variant forms obtained by a variety of methods or by varying greatly the conditions in any one method has led us to concentrate our attention on those simple procedures which first disclosed the type of change for which we were looking. These have been :—

1. Cultivation of the organism at 37°C. in nutrient broth (papain digest of mutton, pH 7·8), to which had been added, in the proportion of 1 part to 9, the requisite mono-specific agglutinating serum. At this dilution of the serum the phenol present did not exert a serious inhibitory effect on growth.

The medium, to economize in the consumption of specific serum, was dispensed in approximate 0·5 c.c. amounts in small tubes (ca. 3″ × 0·3″). The amount of culture inoculated was usually sufficient to confer a faint turbidity to the medium.

When the tubes were incubated for more than 24 or 48 hours the cotton-plugs were impregnated with sterile paraffin wax to prevent evaporation of the medium and, with this, concentration of the phenol.

2. Cultivation of the organism at 37°C. in broth containing activated mono-specific serum : Inoculation was made into a mixture of 1 c.c. of a 1 in 10 dilu-tion of mono-specific agglutinating serum, 0·5 c.c. of a saline 1 in 5 or 1 in 10 dilution of fresh normal guinea-pig serum and 0·5 c.c. of papain digest broth—giving a total volume of 2 c.c. and a final serum concentration of 1 in 20.

After 24 hours' incubation and on succeeding days platings were made from the supernatant fluid in each tube. (Time has not allowed parallel examination of the deposit of agglutinated culture.) The growth appearing on these plates was examined by slide-drop agglutination tests with mono-specific Ogawa and Inaba sera and with a suitable rough serum, all applied in comparable volume to the droplets of emulsified culture in a dilution of 1 in 10 and so acting in a final dilution of about 1 in 20.

Preliminary tests on each plate culture were carried out with a sweep sample of the confluent growth or of many colonies and no attempt was made to study individual colonies until such broad sampling gave an indication of mixed or completely altered agglutinability. Sometimes where the discrete colonies were sufficiently grown, differential slide agglutination tests were carried out on these directly from the primary platings : more usually a number of colonies—the chances being gauged from the behaviour of the sweep sample—were subcultured as macro colonies on a well-dried agar plate for later study. The variant races ultimately selected for preservation and fuller study were replated once or twice and propagated in each case from isolated colonies.

Two phenomena tended to impede the satisfactory application of the method of sweep sampling : Extensive roughening of the cultures with consequent salt agglutinability and the presence in some cultures—particularly those of the El Tor strains—of granular or slimy material, possibly lipoidal, which likewise interfered with confident observation of partial specific agglutination.

It was found that these untoward effects could be eliminated by emulsifying some of the culture in saline—preferably 1·6 per cent NaCl solution—centrifuging down and removing the salt-precipitable material, and then by more vigorous centrifugalization collecting the salt insensitive residue of the culture for serum tests.

Full-scale agglutination tests were made by the usual macroscopic technique using the drop method of dilution and saline suspensions of living agar-grown vibrios. The tubes were dipped in a water-bath at 50°C. for 2 to 4 hours at which term first readings were taken, the final readings being made after the tubes had stood overnight in the refrigerator.

In testing the agglutinin-absorbing powers of parent and variant races the living vibrio cultures, scraped from agar, were directly emulsified in the agglutinating sera suitably diluted and the reaction mixtures were allowed to stand for one hour at 37°C. before being clarified in the centrifuge.

In any agglutination and agglutinin-absorption tests involving 'R' variants or partially rough forms the saline concentration in all menstrua was reduced from the usual 0·85 per cent of NaCl to one-half or one-third of that amount.

OBSERVATIONS ON TRANSITION FROM THE OGAWA TO THE INABA TYPE.

The essence of our findings on this point are readily stated : from all our 10 strains of *V. cholerœ* type Ogawa and three strains of Ogawa type El Tor vibrios we have isolated cultures which when tested with the type mono-specific sera and with 'R' serum have reacted exclusively and wholeheartedly with the serum

of the Inaba type. Such of these presumed 'Inaba variant' cultures as have been studied by fully expanded agglutination and agglutinin-absorption tests against Ogawa and Inaba type antisera—whether natural unabsorbed sera or mono-specific sera purged of common agglutinin—have reacted precisely like strains of the Inaba type (accepting the strain 'Inaba' as prototype of the sub-group). Though anti-sera have not yet been raised against the variant cultures, permitting the counter test to be performed, we have no positive reason to doubt that serologically they are typical races of that type.

Lest it be thought that we too readily or uncritically accept these races of Inaba-reacting vibrios, isolated from Ogawa-labelled cultures, as true derivatives of the Ogawa type be it pointed out that the likelihood that all our 13 cultures of the Ogawa type were infected from first isolation or during laboratory passage with an Inaba contaminant is relatively small; that we have not, apart from the specific treatment described, been able to discover signs of duality in the original strains; that isolation of the presumed variants was, in the case of all the *V. choleræ* type Ogawa strains studied, at least once, sometimes twice or three times, accomplished from fully tested races sprung from a carefully selected discrete colony; and that we may confidently assert that the reagents involved in the experiments were bacteriologically sterile.

It would be difficult and of little profit to detail in sequence the various experiments made. Table I gives in summary the results of some of the more important and successful trials in respect of which details have been preserved and in Table II are set out the results of an experiment made to compare the serological properties of one of the variant cultures with those of its parent culture.

<div align="center">

TABLE I.

Showing the agglutinative response of culture samples after treatment of V. choleræ *Ogawa type strains with mono-specific Ogawa antiserum (agglutinating titre of undiluted serum* 1 : 500 *to* 1 : 1,000).

</div>

Strain.	Experiment.	Method of treatment.	Day of examination.	AGGLUTINATIVE PROPERTIES OF SWEEP SAMPLE WITH MONO-SPECIFIC SERUM.			NUMBER OF COLONIES EXAMINED REACTING AS		
				Ogawa.	Inaba.	Rough.	Ogawa.	Inaba.	Rough.
M.L. 15	A	Ogawa serum 1 : 10	1	+++	—	—	...	...	...
			2	++	++	t	5	5	1
	B	,, ,, 1 : 10	1	t	t	+++	1	—	10

Explanation :— +++, ++, + and t indicate varying degrees of agglutination from complete to traces.
    ... = No colonies examined.
    — = Negative finding.

TABLE I—*contd.*

Strain.	Experiment.	Method of treatment.	Day of examination.	AGGLUTINATIVE PROPERTIES OF SWEEP SAMPLE WITH MONO-SPECIFIC SERUM.			NUMBER OF COLONIES EXAMINED REACTING AS		
				Ogawa.	Inaba.	Rough.	Ogawa.	Inaba.	Rough.
M.L. 66	A	Ogawa serum 1:10	1	+	++	*n*	1	7	—
	B	Ogawa serum 1:20	1	+++	—	—	...	...	...
		Complement 1:20	2	—	+	+++	—	1	3
		Ogawa serum 1:20	1	*n*	*n*	+++	...	...	...
		Complement 1:40	2	—	+	+++	—	1	3
	C	Ogawa serum 1:10	1	+	+++	—	1	10 (1 Mixed).	
	D	Ogawa serum 1:20	1	++	++	++	3	(9 Intermediate).	
		Complement 1:32							
2586	A	Ogawa serum 1:10	1	—	+++	—	—	6	—
358/203A	A	Ogawa serum 1:10	1	++	+++	+	1	2	—
368/213B	A	,, ,, 1:10	1	+++	+	+	3	1	—
534/380A	A	,, ,, 1:10	1	++	++	—	3	3	—
536/382C	A	,, ,, 1:10	1	+++	++	+	3	1	—
551/397C	A	,, ,, 1:10	1	+++	*t*	*t*	7	—	—
			2	+++	*t*	+	10	—	—
843/694B	A	,, ,, 1:10	1	+++	+	+	14	1	—
			2	+	++	++	1	7	2
358/203A	B	Ogawa serum 1:10	1	*t*	+++	*t*	—	10	1
368/213B	B	,, ,, 1:10	1	++	+	++	2	2	5
534/380A	B	,, ,, 1:10	1	+	+++	—	2	8	
551/397C	B	,, ,, 1:10	1	*t*	+++	*t*	—	12	—

Explanation:— +++, ++, + and *t* indicate varying degrees of agglutination from complete to traces.
... = No colonies examined.
— = Negative finding.

TABLE I—*concld.*

Strain.	Experiment.	Method of treatment.	Day of examination.	AGGLUTINATIVE PROPERTIES OF SWEEP SAMPLE WITH MONOSPECIFIC SERUM.			NUMBER OF COLONIES EXAMINED REACTING AS		
				Ogawa.	Inaba.	Rough.	Ogawa.	Inaba.	Rough.
551/397C	C	Ogawa serum 1 : 20 Complement 1 : 20	1 & 2	+++	—	+	...	...	...
		Ogawa serum 1 : 20 Complement 1 : 20	1	+++	++	++	3	2	7

Explanation :— +++, ++, + and *t* indicate varying degrees of agglutination from complete to traces.

... = No colonies examined.

— = Negative finding.

TABLE II.

Comparison of the agglutinative and agglutinin-binding properties of strain M.L. 66 (type Ogawa) with those of the culture M.L. 66 (Inaba variant race 12) derived from it.

Strain tested for agglutination.	TITRE LIMITS IN AGGLUTINATION TESTS WITH	
	Ogawa antiserum (*versus* M.L. 66).	Inaba antiserum (*versus* 569B).
	Before absorption.	
Parent M.L. 66 (Ogawa) ...	2,000	5,000
Inaba (Tokyo)	1,000	5,000
M.L. 66 (Inaba variant) ...	1,000	10,000
	After absorption with parent strain M.L. 66 (Ogawa).	
Parent M.L. 66 (Ogawa) ...	< 100	< 100
Inaba (Tokyo)	< 100	1,000/2,000
M.L. 66 (Inaba variant) ...	< 100	2,000
	After absorption with M.L. 66 (Inaba variant).	
Parent M.L. 66 (Ogawa) ...	2,000	< 100
Inaba (Tokyo)	< 100	< 100
M.L. 66 (Inaba variant) ...	< 100	< 100

From Table I it is seen that the type of variation described was demonstrated at the majority of the trials presented. In these the C.R.I. mono-specific Ogawa type antiserum was employed : similar success was achieved with our own preparation of mono-specific Ogawa serum. Setting aside those trials at which generation of Inaba-reacting vibrios was not proven—and the number of these failures might have been reduced by more leisured and assiduous study—the extent of the change observed in the treated culture was very variable; sometimes Inaba-reacting colonies were relatively few in number in platings and difficult to find even where the behaviour of the sweep sample gave good reason to suspect their presence; at other times there was a serological landslide in the composition of the supernatant culture and the residue of the parent type was as difficult to detect. The precise degree of the response could not be forecast; the individual strains behaved very differently at successive trials, now showing in platings an occasional Inaba type colony, now showing an overwhelming majority of such colonies, now failing to show discernible change other than, perhaps, roughening.

Collaterally with the appearance of Inaba-reacting vibrios in the serum treated Ogawa cultures there was a tendency to the development of rough variants particularly when the serum employed was activated with complement. The presence of numerous rough forms in platings placed, as has been noted above, difficulties in the way of exact observation of the serological properties of the smooth constituents of the culture. On the whole, however, we have been relatively little hindered in our examination of Ogawa cultures by the occurrence of extensive roughening in the plated culture samples.

When the fact of change from Ogawa to the Inaba type was first demonstrated we regarded it as probable that various transitional forms would, from time to time, appear in our cultures. Save on one occasion, this expectation has not been realized in experience : indeed the completeness of the serological change observed has seemed a striking feature of the phenomenon and where colonies with dual serological behaviour have been encountered, as they have been frequently enough, they have readily been shown by replating to have been mixtures of distinct Ogawa-reacting and Inaba-reacting elements.

The single instance in which forms of intermediate behaviour were detected was an experiment (included in Table I) in which the Ogawa strain M.L. 66 was cultivated in activated Ogawa type mono-specific serum. Platings made on the day of incubation showed, with a few colonies of the parent type and others frankly rough, a number which yielded in subculture races which, while not appreciably clumped by salt in physiological concentration, reacted fully not only with Ogawa and Inaba mono-specific sera but also with rough antiserum. Replating has failed to resolve these races into more specifically reacting colonies and we are of opinion that in them the units actually present trivalent agglutinative properties: that they are transitional forms slightly affected by roughening. In absorption tests performed they entirely exhausted the rough serum employed and reduced the homologous titres of the mono-specific Ogawa type and Inaba type sera (in both cases about 1 : 1,000) to a small residue (in both cases about 1 : 100 to 1 : 200). It may be noted that all or almost all the typical 'Inaba variant' colonies examined were quite smooth. Whether the peculiarity of these races was in any way due to the activation of the antiserum, it is impossible to say. In demonstrating change

to the Inaba type we have not on the whole found any advantage in adding complement to the medium.

OBSERVATIONS REGARDING TRANSITION FROM THE INABA TO THE
OGAWA TYPE.

Our findings on this issue lend themselves rather less readily to direct and confident statement than to those presented above : first, because the material on which we have worked has proved serologically complex ; second, because negative results where obtained—and obtained under conditions of limited experimental range—cannot be regarded as entirely conclusive.

The strains which we selected, on the basis of catalogued type, from the C.R.I. collection to serve as representatives of the Inaba type were found on examination with our mono-specific sera to fall into two groups. The first group was composed of the original Inaba strain and the strains 191A, 194A, 569B and Khulna I, none of which in slide-drop agglutination tests were affected at a 1 in 10 or higher dilution by the mono-specific Ogawa antisera which we employed and likewise gave completely negative results with the same sera in water-bath tests at dilutions of 1 in 20 upwards. These we regard as representing the Inaba type proper. To this group belonged also the El Tor strains D13, D25 and D26.

The second group was formed by the remaining strains (370-215A—953-806C) all relatively recently isolated in Calcutta. These strains while reacting fully with our Inaba mono-specific sera showed also definite though partial agglutination with our mono-specific Ogawa sera—sera prepared by treatment of the ' whole ' antiserum with classic Inaba strains. In slide-drop agglutination tests mono-specific Ogawa serum diluted 1 in 10 to 1 in 20 caused considerable immediate clumping of the suspensions, leaving always, however, a persisting turbidity. Such efforts as we have made to separate strongly Ogawa-reacting and weakly Ogawa-reacting races from these cultures have not been successful though it is quite clear that in them the units are of very varied sensitivity to Ogawa agglutinins.

In fuller agglutination tests by the water-bath technique the Ogawa cross reaction has seemed rather less striking, but what is important is that in absorption tests the cultures in question are able to reduce the specific titre of Ogawa mono-specific serum to one-fourth or one-third of its original value—though they are unable to exhaust it further when the absorption procedure is repeated. It would seem, then, that these alleged Inaba cultures contain some part of the specific Ogawa serological complex, though not that complex in entirety.

Though the agglutination of the cultures by mono-specific Ogawa sera is relatively weak, their behaviour in absorption experiments coincides precisely with Nobechi's description, as expressed in his often reproduced table of agglutinin cross-absorption tests, of the properties of the Intermediate type, with its fully-developed Inaba antigen and qualitatively imperfect Ogawa antigen. We have little doubt that all the strains in question belong to this Intermediate fraternity.

With these explanations the way is cleared for a statement of our results. Dealing first with the eight cultures, 5 *V. cholerae* and 3 El Tor, adhering to the classic Inaba pattern, we have failed, despite repeated trials, to isolate from

any one of these, by cultivation in the presence of the homologous mono-specific serum, races agglutinable by mono-specific Ogawa serum. Platings made after 24 hours' incubation of the primary cultures almost invariably showed ' R ' colonies and often ' R ' colonies almost exclusively ; in those made after 48 hours complete roughening was the rule. The predominance of ' R ' forms in our cultures of Inaba strains was in contrast to our experience with the Ogawa type, where their appearance tended to be delayed and where it was relatively seldom, save where complement was employed, that they became the sole or even dominant components of the supernatant broth culture.

The case of the Calcutta strains of intermediate serology turned out differently. Here, too, rough variants appeared early and in abundance in the broth cultures but from four (370-215A, 389-234B, 794-645A, 953-806C) of the eight strains (each of which was tested 3 to 5 times) we were able to cultivate, though in each instance on one occasion only, races indistinguishable in agglutination and absorption tests from strains of the Ogawa type.

On the four occasions on which this serological change was observed the variant became the dominant component of the supernatant broth culture and was readily isolated from platings. In the case of strain 370-215A, the ' Ogawa variant ' was obtained from a carefully tested race derived from a single colony : in the remaining cases the isolation was made from the cultures as received.

Since examples of the method of recognition and identification of variant cultures have been given in Tables I and II to cover the case of the ' Inaba variant ' forms we have not regarded it as necessary to include here formal tables of the tests, essentially similar in principle and equally conclusive, made with these ' Ogawa variants '.

DISCUSSION.

It is relatively seldom that the bacterial serologist, so often under the stigma of bringing complexity to taxonomy hitherto straightforward, finds himself, as we at the moment do, in the happy position of urging a simplification of ideas. The unequivocal indication of the observations described above is that the system of subsidiary classification of *V. cholerœ* at present in vogue—and in the popularization of which one of us has played some part !—is, whatever may subsequently prove to be its practical importance, taxonomically invalid. Without denying the possibility that on some other basis true serological subtypes of the classic vibrio may yet be found, it can be said that, whether one chooses to speak in terms of Ogawa, Hikojima and Inaba types or like Burrows and his collaborators in terms of a factorial system, the extreme probability is that one is expressing merely the range of natural variation of a single organism. Kabeshima in launching the series of observations which have led to the existing doctrine of the types had grasped, however slender his evidence, the true nature of their relationship.

That the Inaba type may be readily derived from the Ogawa type now seems to be conclusively proved. On the other hand, the possibility of the reverse change from Inaba to Ogawa cannot be decided summarily on the limited body of negative evidence we are able to present. It may be that under rather different conditions of serological treatment or by some other form of stimulation the Inaba type may

be induced to revert. We do feel, however, that the contrast in the behaviour of the two types under very comparable conditions of treatment is sufficiently marked to imply the probable significance of the negative findings in the instance of the Inaba strains—all the more so in that those cultures, originally mistaken for Inaba strains, which contained a tangible if qualitatively imperfect remnant of Ogawa antigen did not prove intractable under the treatment we applied.

It is always interesting and sometimes profitable to speculate as to the nature and origins of such phenomena of biological change. If we accept at face value the evidence which suggests that, so far as specific serological intervention is concerned, the change from Ogawa to Inaba is irreversible and gamble on the knowledge that, in the bacterial world as elsewhere in living nature, variations of loss and degradation overwhelm in number those of gain and synthesis, we arrive at the provisional thesis, advanced without prejudice to future correction :—

That the Ogawa serological complex represents the known acme of elaboration of the specific somatic antigen of *V. choleræ.*

That this antigen is subject to degradation presumably by failure of the organism to synthesize certain chemical groupings.

That this change is expressed *serologically* as a positive modification in the detail of the antigen and not merely as a factorial loss.

That from this debased Inaba antigen there is no easy return to the Ogawa state by a revival of lost synthetic power, the only escape from the interference of specific antibodies being in ' rough ' change, i.e. entire failure to synthesize the specific complex with resultant unmasking of the ' R ' antigen.

It is probable that proof or disproof of this hypothesis can be obtained only by chemical means.

There is little in our work to show how the basic variation is engendered. One possibility is of course that the serous attack is a direct stimulus to change. It is, however, a little difficult to understand how an attack directed against the superficies of the cell—against what is probably largely in the nature of a secretion—could cause transmissible modification of this or of the underlying germinal substance on which its synthesis depends. Even if one is prepared to be frankly teleological and assume that the cell in its wisdom takes requisite evasive action one is left to explain why only one cell in a myriad is thuswise.

The other line of possible explanation is that the variation in question occurs naturally in the multiplication of the Ogawa culture but too rarely to be commonly discernible in laboratory studies, being only exposed, where present, when the teeming mass of unaltered culture is precipitated leaving the lone variant to colonize without competition the supernatant medium. If the change to Inaba is indeed, as we have suggested, one of synthetic defect it is readily conceivable that such forms might arise—as do ' R ' forms and other loss variants throughout the living world—during the multifold divisions of cells and presumably through some fault in germinal distribution. Such a conception of occasional chance variation would be compatible with the apparent fortuity of success and failure in our efforts to isolate the Inaba type from Ogawa cultures.

SUMMARY.

Various strains of cholera and El Tor vibrios were cultivated in broth containing, with and without the addition of complement, the type-homologous monospecific antiserum and then examined for signs of serological change.

From 10 strains of *V. cholerœ* and 3 strains of *V. El Tor* belonging to the Ogawa type, and grown in the presence of Ogawa mono-specific antiserum, there were isolated in each case races serologically indistinguishable from strains of the Inaba type.

When similarly exposed to the action of mono-specific Inaba antiserum, 4 of 8 strains of *V. cholerœ*, predominantly Inaba-like in serology, but possessed of an imperfect Ogawa factor, yielded cultures of the Ogawa type.

On the other hand 5 strains of *V. cholerœ* and 3 strains of *V. El Tor* adhering strictly to the Inaba type failed, when cultivated in the homologous mono-specific serum, to show serological change other than roughening.

The significance and nature of the phenomena are discussed and it is pointed out that the existing classification of the vibrios of ' O ' group I into serological types is taxonomically invalid.

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STATISTICAL EVALUATION OF ANTI-CHOLERA INOCULATION AS A PERSONAL PROPHYLACTIC AGAINST CHOLERA AND ITS EFFICACY IN THE PREVENTION AND CONTROL OF CHOLERA EPIDEMICS.

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INTRODUCTION.

For many years cholera vaccine has been extensively used in India and elsewhere as a personal prophylactic, especially when cholera epidemics are imminent or in progress. Based on the experience of mass inoculation campaigns carried out during epidemics, the value of the vaccine in affording protection against cholera has been studied by Russell (1927) in Madras, Govinda Raju and Sarkar (1931) in Bengal. Millar and Ghulam Mohi-ud-din (1937) in Kashmir, and Bozman and Lewis (1936) in Burma. Excepting in the first of the above studies. there appear to be doubts about the effective exposure to the disease being identical in the case of the inoculated and uninoculated populations. Further. as the existence of serological types among cholera vibrios had not been sufficiently recognized at the time of the above studies. the types of vibrio included in the vaccines used for inoculation cannot now be stated with certainty.
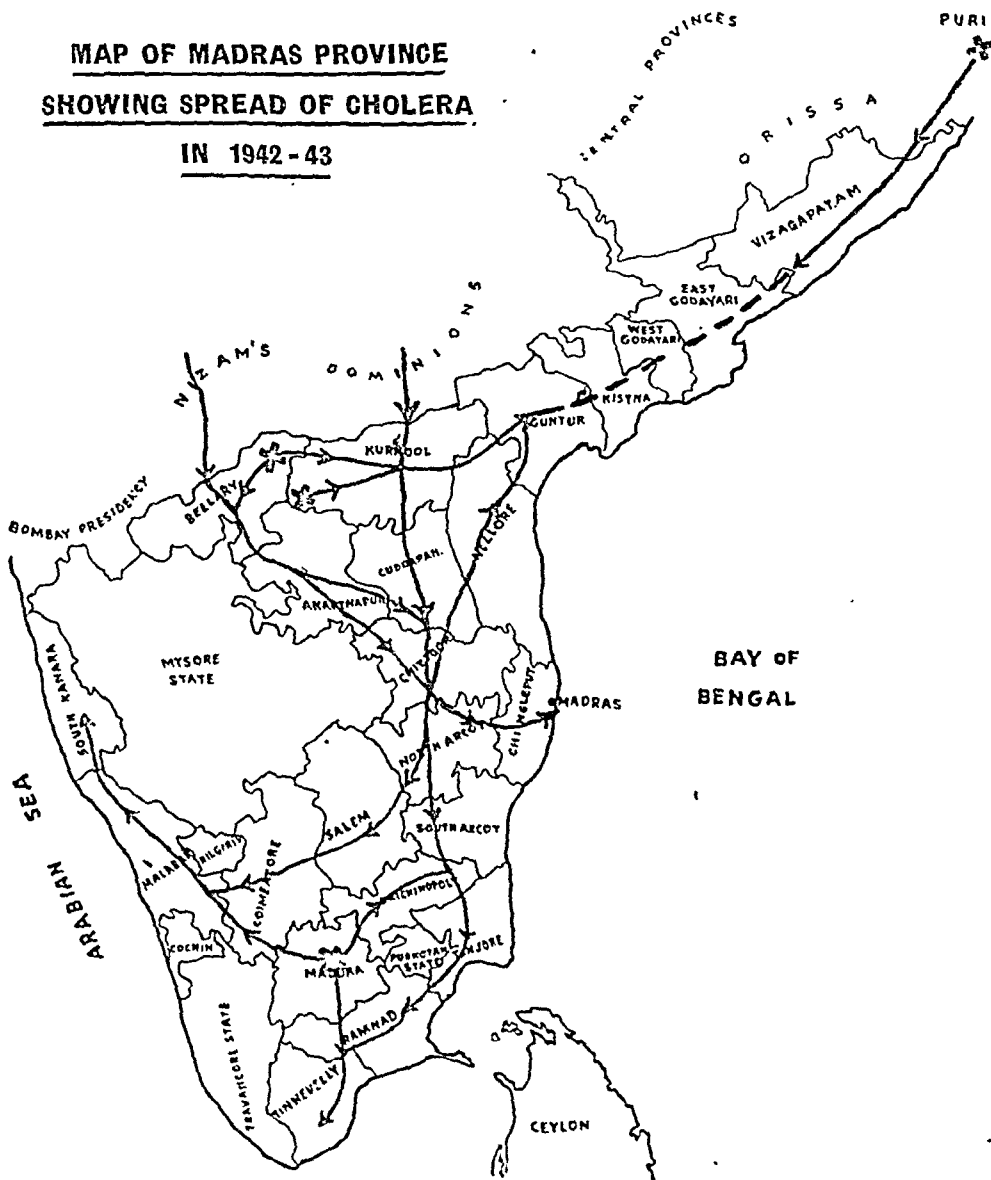
Furthermore, apart from the value of the vaccine as a personal prophylactic, other immunological problems, such as the duration of immunity conferred and the case fatality rate in inoculated subjects, had not been considered by the above authors. For these reasons, the large number of inoculations done during the widespread and severe epidemic of cholera which, in 1942 and 1943, involved over two-thirds of the Madras Province, was considered to afford an exceptional opportunity to re-assess the value of inoculation in the prevention and control of epidemics, and also to study the time factor in the development of immunity and the period for which it remains effective.

The epidemic of 1942-43 in Madras was one of the six-yearly periodic waves characteristic of cholera in this province (Russell and Sundararajan, 1928). As in the major epidemics in the past, infection was imported through Bellary and Kurnool districts, cases occurring more or less simultaneously in both these districts late in the first quarter of 1942. Conditions of famine and acute water scarcity present at the time in these districts were particularly favourable for the persistent prevalence of infection. A few weeks later, cholera broke out among the visitors at two pilgrim centres, one in the Kurnool and the other in the Bellary district. Pilgrims returning from these festivals spread the infection to the villages through which they passed, thereby creating multiple foci of infection in the other ceded districts. The infection spread subsequently to the south and south-eastern districts of the province. The festival centres at Palni in Madura district and Puri in Orissa were additional foci from which infection radiated into the adjoining districts. In the course of a few months, approximately two-thirds of the province was involved, the north-east coastal areas alone escaping serious infection. The routes along which the infection spread are indicated in the Map (*see* opposite).

The following extract from the report of the District Health Officer of South Arcot will be found to be of epidemiological interest :—

'Generally, in all the taluks, there were two distinct rises in the trend of the epidemic. The first phase was within three weeks from the date of onset and another along with the usual winter epidemic, commencing from the middle of December 1942 to the first half of January 1943. The second phase of the epidemic is the result of the part played by the groundnut pickers (migrating agricultural labourers). The labouring classes who leave their homes in search of livelihood to taluks where groundnut produce is in harvest and return home by the end of December, carried with them the infection. Further, these labourers spend their time in groundnut fields, eat freely raw groundnuts and take water from any source available and sleep in the open air and expose themselves to the inclemencies of the weather. To quote one instance, cholera infection in Villupuram taluk was confined to villages adjoining the river Pennar and it was subsiding. But on 16th November, 1942, there was a sudden outbreak of cholera in one of the villages in a gang of groundnut pickers. These labourers fled away in panic in different directions while suffering from or incubating the disease. Information was received by this department only when the labourers had run away. This gang was responsible for spreading the infection in 22 villages in Villupuram taluk and in addition caused infection to be spread in Gingee taluk which had been free from the epidemic during all these months.'

MAP OF MADRAS PROVINCE
SHOWING SPREAD OF CHOLERA
IN 1942-43

PURI

CENTRAL PROVINCES

ORISSA

VIZAGAPATAM

EAST GODAVARI

WEST GODAVARI

NIZAM'S

DOMINIONS

KISTNA

GUNTUR

KURNOOL

BOMBAY PRESIDENCY

BELLARY

CUDDAPAH

NELLORE

ANANTAPUR

MYSORE STATE

CHITTOOR

CHINGLEPUT

MADRAS

BAY OF BENGAL

NORTH ARCOT

SOUTH ARCOT

SALEM

SOUTH KANARA

MALABAR

NILGIRIS

COIMBATORE

TRICHINOPOLY

ARABIAN SEA

COCHIN

MADURA

PUDUCTAI STATE

TANJORE

RAMNAD

TRAVANCORE STATE

TINNEVELLY

CEYLON

During the 1942-43 epidemic approximately 300,000 cases of cholera of which 169,400 were fatal were recorded. In all, about 12·48 million persons received anti-cholera inoculation.

PRESENT INQUIRY.

The present inquiry deals mainly with the statistical evaluation of the data collected during and after the epidemic of cholera in several affected areas. For an inquiry of this kind, the most suitable data would be those secured on an experimental basis relating to a group of persons in a community inoculated prior to the expected occurrence of cholera with uninoculated persons serving as controls. For obvious reasons, the two groups of population should be comprised of sufficiently large numbers and should be more or less identical in every respect except that one group is inoculated and the other not. There are, however, certain difficulties in getting such material for study. In the first place, it is only when cholera actually occurs in a village that the people are willing or can be persuaded to accept inoculation. Secondly, when, at the time of an actual or threatened epidemic in a village, there is a demand for inoculation, it would be administratively unsound to restrict inoculation to some persons in the community and deny it to others for the purpose of an experimental study, especially when it is the accepted policy of the public health administration to persuade everyone to accept inoculation. Thirdly, the present inquiry was contemplated only after thousands of inoculations had already been done. By this time, the epidemic had become widespread and the available field staff and vaccine supplies were barely sufficient to meet the needs of the areas actually affected by cholera : it was impossible to release personnel and vaccine for use in other areas in anticipation of the occurrence of cholera for the purpose of a planned inquiry. Certain data, virtually equivalent to anticipatory inoculations, were, however, obtained in the present investigation. In the course of the epidemic, several hundred villages experienced two or more outbreaks of cholera at varying intervals. The inoculations done at the time of the first outbreaks in these villages may, therefore, be regarded as anticipatory inoculations with respect to the second and subsequent outbreaks.

The objects of the inquiry have been to determine :—

(*a*) Whether anti-cholera inoculation confers immunity against cholera, and if it does, the time taken for its development from the date of inoculation. The data required to determine these points are the relative incidence of cholera in the inoculated and the uninoculated groups of the population exposed to risk, and the incidence of cases on the days succeeding the date of inoculation.

(*b*) The duration of the immunity conferred by inoculation. This could be determined from the incidence of cholera among the inoculated persons who continued to be exposed to risk in the course of an outbreak of long duration and in the populations at risk in villages which experienced more than one outbreak of the disease when the interval between such outbreaks was not unduly short.

(c) The influence, if any, of mass immunity on the occurrence of subsequent outbreaks. This could be determined from the data in (b).

DEFINITIONS AND CRITERIA.

For the purpose of the present study, the following definitions or criteria have been adopted :—

(i) As varying periods are required for the development of a sufficiently high level of immunity after prophylactic inoculations, the period of six days prescribed in the International Quarantine Regulations was adopted as the period necessary to confer protection in the case of anti-cholera vaccine. A person inoculated with anti-cholera vaccine was deemed to be ' protected ' after the sixth day of inoculation.

(ii) In the case of villages which experienced more than one outbreak of cholera, the outbreaks were considered as separate and distinct if the interval between them was 30 days or more. This period was considered necessary in order to avoid any fallacies that might arise on account of missed or sub-clinical cases. Alternative classifications with intervals of two weeks and three weeks were tried in 163 villages in Villupuram range in South Arcot district. The results obtained from this classification did not show any material differences from those obtained by the classification which was finally adopted.

(iii) The duration of an epidemic in any given place is defined as the number of days, both inclusive, from the date of the first to the date of the last case which occurred during the outbreak.

(iv) Population at risk : in a disease such as cholera, especially during a widespread epidemic, the risk of contracting the disease is not confined solely to case contacts or to residents in the immediate vicinity. The population at risk has to be judged on epidemiological consider-ations. Among the factors contributing to the spread of infection, the most important is drinking water supply. In the villages under survey, as in the other rural areas of the province, the principal sources of drinking water are wells, rivers, canals, irrigation channels, tanks and ponds, and these are used not only for drinking purposes but also for bathing and washing. The washing of infected clothing and other materials in or near such sources is not uncommon. The contamination of the different sources is, therefore, only a question of degree. As regards other aspects of environmental sanitation influencing the spread of infection, only the larger villages with semi-urban characteristics have even a rudimentary organization for conservancy and scavenging, while there is a complete lack of such facilities in the smaller villages. Arrangements for institutional segregation of cholera cases do not exist in rural areas. The social custom of relatives and friends congregating at funerals and partaking of common meals thereafter is yet another import-ant factor. In these circumstances, to limit the boundaries

of an infected area to a house or street where cholera has occurred. or to the locality within a specified distance. would not only be to ignore epidemiological considerations but to introduce considerable complications as well. Accordingly. in the present survey, if cases of cholera had occurred in the village proper as well as in the hamlets (*vide infra* for description) the census population of the whole village was taken as the population at risk. If the cases were restricted to one or more hamlets only, the population of the hamlet was taken as the population at risk ; where the census figures of the population of individual hamlets were not available this was determined by rough enumeration.

TYPE OF CHOLERA VACCINE AND ITS ADMINISTRATION.

The serological type of *V. cholerœ* prevalent in different outbreaks in the several districts of the province has been under continuous study since 1936 by one of us (K. V. V.) at the King Institute. Guindy. As a result of the examination of nearly 4,000 strains of *V. cholerœ* during this period, it has been found that. while in the earlier years there was a clear demarcation of areas of prevalence of Inaba and Ogawa types, in all outbreaks which have occurred since 1940 the Ogawa type has predominated throughout the province. However, in accordance with the standard prescribed for cholera vaccine in India (Taylor, 1941) both types of *V. cholerœ* were included in the prophylactic vaccine prepared at the King Institute, Guindy. which was used throughout the present investigation.

The greater proportion of the inoculations were done by Sanitary Inspectors trained in inoculation work ; only a small proportion was performed by the staff of medical institutions or by private medical practitioners.

The vaccine was almost invariably administered in a single dose as this alone is feasible in mass inoculation campaigns during epidemics. In the case of adults, the dosage was 1 c.c. of vaccine containing 8,000 million vibrios ; proportionately smaller doses were given to children.

COLLECTION AND COMPILATION OF BASIC DATA.

For a proper understanding of the methods of collection and compilation of the data and of the discussions on the inquiry, a brief description of the system of Public Health Administration with special reference to notification, prevention and control of communicable diseases is given below.

The present investigation relates to rural areas only. Public Health Administration in these areas is closely linked with the Revenue Administration. The Executive Head of the Government for Revenue Administration in each of the twenty-five districts in the province is the Collector. Every district is divided into smaller geographical units called taluks. there are about 8 to 12 taluks in each district. The taluk revenue official is the Tahsildar. Each taluk comprises a number of villages. ranging from 50 to 200 or more. Each village is composed of a main central part and a number of suburbs which in this province are designated

The total number of villages and hamlets for which data have been compiled is 2,350. The population of these villages was approximately 3·3 millions of whom 1·18 millions were inoculated. About 55,000 cases and 30,000 deaths from cholera were recorded in these villages during the epidemic. *The data included in the study relate, however, only to the period after the commencement of inoculation.* A summary of the basic data is given below :—

(*a*) *i.* Total number of villages and hamlets included
  in the survey   2,350

    ii. Number of villages and hamlets which had
  two or more outbreaks in the epidemic ...   627

(*b*) *i.* Total population of the 2,350 villages included
  in the survey   ...   . 3·30 millions.

    ii. Number of inoculations in above   ... 1·18   ,,

(*c*)

	PROTECTED.		UNINOCULATED.		TOTAL.	
	Population.	Cholera cases.	Population.	Cholera cases.	Population.	Cholera cases.
Whole experience ...	709,977*	1,118	2,119,568*	34,336	2,829,545	35,454
Second and subsequent outbreaks.	281,484	241	541,808	6,580	823,292	6,821

* Weighted mean population.

In a field survey of this magnitude, the scrutiny of the particulars of nearly 35,500 cases in the village cholera registers among 1·18 million entries in the inoculation registers of the Health Inspectors, and the transference of the relevant information to the posting cards, necessitated the maintenance of a very large staff. The work was subjected to check at every stage in order to ensure accuracy. As a further precaution, the basic data were subjected to the closest scrutiny by an independent observer, namely, Dr. Chandra Sekar, Professor of Statistics in the All-India Institute of Hygiene & Public Health, Calcutta, who found that such omissions and inaccuracies as were detected were of a minor nature and inconsequential.

STATISTICAL ANALYSIS OF THE DATA.

According to Greenwood and Yule (1915) statistics relating to inoculated and uninoculated persons should satisfy the following requirements :—

(*a*) The persons must be in material respects alike : they must not differ in age, sex, social or racial constitutions.

(b) Effective exposure to the disease must be identical in the case of the inoculated and uninoculated persons.

(c) The criteria of the fact of inoculation and the fact of the disease having occurred must be independent.

Although the populations in the villages covered by the survey were composed of several castes and communities with varying standards of personal and environmental hygiene which are recognized factors of importance in the causation of cholera, there was no reluctance on the part of persons belonging to any particular caste or community to accept inoculation. Further, there were no objections to inoculation on the score of age, sex or social status. There being no selectivity of any kind in the matter of inoculations, the first of the conditions may be considered as satisfied.

As regards the second condition. only cases of cholera which occurred after the commencement of the inoculation campaign have been included in the present study. The effective exposure of the inoculated and uninoculated persons to risk of infection is therefore identical.

The present inquiry was not initiated until late in the epidemic when the bulk of the inoculations had already been done and the cases of cholera included in the survey had occurred. There was, therefore, no bias in selecting any specified population groups for the study. Secondly. the inoculation registers were not available to the village Headmen who made preliminary inquiries as to whether the cases recorded in their cholera registers were inoculated or not inoculated : the information which they gathered from the householders was subsequently checked by the Health Inspectors by inquiries as well as by comparison with the inoculation lists : a test check was also done by the District Health Officers : further. it is the experience of field workers that the occurrence of cholera among inoculated persons is given special prominence and suppression of information is very rare. In these circumstances, the third condition laid down by Greenwood and Yule *(loc. cit.)* about the independence of the fact of inoculation and the attack of cholera may be considered to have been satisfied.

WHETHER ANTI-CHOLERA INOCULATION CONFERS IMMUNITY.

If anti-cholera inoculation affords protection. the incidence of cholera in the protected population in a community exposed to the risk of infection should either be *nil* or be significantly lower than in the uninoculated persons in the community. For this examination, two sets of data of the comparative incidence of cholera in the protected and uninoculated persons are available. namely (a) the cases, from the commencement of inoculations to the end of the epidemic, in all the 2,350 villages included in the survey, and (b) the cases in the second and subsequent outbreaks in 627 villages which experienced more than one outbreak.

As regards the former. it is necessary to point out that. in the great majority of villages. all inoculations were not done on a single day but were spread out over

a number of days. The proportion of the ' protected ' to the uninoculated persons varied, therefore, from day to day and the period of exposure of these two groups to risk of infection in the course of the epidemic was changing. In order to make allowance for this, the weighted means of the protected and uninoculated persons were taken as the populations at risk in each group, the weights used being the days of exposure to infection. The aggregate weighted mean ' protected ' population was 709,977 and the weighted mean uninoculated population was 2,119,568. For assessing the prophylactic value of anti-cholera inoculation, the experience of the inoculated persons within the first six days after inoculation has been excluded in order to avoid the fallacies that might otherwise arise as a result of partial immunity.

As regards the cases in the second and later outbreaks, the experience is restricted to persons who had been inoculated in connection with the first outbreak. As defined above, a minimum interval of 30 days must have elapsed before considering a second or subsequent outbreak of cholera as separate and distinct from the preceding one : this ensures the establishment of substantial immunity among the inoculated persons when exposed to risk of infection in the second and later outbreaks. These inoculations are virtually tantamount to anticipatory inoculations in a planned investigation and the conclusions drawn from these data should be more sensitive than those in the previous case.

The data relating to the experience from the commencement of the inoculations to the end of the epidemic and in the second and subsequent outbreaks are analysed in Table I :—

TABLE I.

Incidence of cholera in inoculated and uninoculated populations.

	UNINOCULATED POPULATION AT RISK.			PROTECTED POPULATION AT RISK.			Ratio of Col. 7 to Col. 4.
	Total.	Cases of cholera.	Rate per 1,000.	Total.	Cases of cholera.	Rate per 1,000.	
1	2	3	4	5	6	7	8
Whole epidemic period.	2,119,568	34,336	16·20	709,777	1,118	1·57	1 : 10·3
Second and subsequent outbreaks.	541,808	6,580	12·14	281,484	241	0·86	1 : 14·1

It will be seen from column 8 of Table I that the incidence of cholera in the uninoculated group is 10 to 14 times greater than in the protected group. The

statistical significance of the figures in Table I is examined below by the χ^2 (Chi-square) test [*vide* Table II (*a*) and (*b*)] :—

TABLE II.

(*a*) *Whole experience.*

Population at risk.		Not attacked.	Attacked.	Total.	χ^2	P
Protected ...	...	708,859	1,118	709,977	} 9,135·09	<0·0016
Uninoculated	...	2,085,232	34,336	2,119,568		
TOTAL ...	...	2,794,091	35,454	2,829,545	...	...

(*b*) *Experience in second and later outbreaks.*

Population at risk.		Not attacked.	Attacked.	Total.	χ^2	P
Protected ...	..	281,243	241	281,484	} 2,875·05	<0·0016
Uninoculated	...	535,228	6,580	541,808		
TOTAL ...	...	816,471	6,821	823,292	...	...

The χ^2 and the corresponding P values are significant in both sets of date [Table II (*a*) and (*b*)] and confirm the findings arrived at from the figures in Table I that inoculation affords substantial protection against cholera.

The differences in the cholera incidence rates in the protected and uninoculated groups are not due to any peculiarities in the age and sex distributions of the cases of cholera. This will be evident from the data relating to 100 villages chosen at random according to Tippets Random Numbers (Table III) :—

TABLE III.

Age and sex distribution of cholera cases in 100 villages selected at random.

	NUMBER OF ATTACKS.			PERCENTAGE TO TOTAL IN THE AGE PERIOD.			PERCENTAGE OF POPULATIONS TO TOTALS.		
	Male.	Female.	Total.	Male.	Female.	Total.	Male.	Female.	Total.
Under 5 years ...	121	106	227	11·5	9·6	10·6	14·2	14·6	14·1
Under 5-14 years	237	276	513	22·6	25·1	23·9	24·8	23·7	24·2
15 years and above	691	718	1,409	65·9	65·3	65·5	61·0	61·7	61·4
TOTAL ...	1,049	1,100	2,149	100	100	100	100	100	100

It will be seen from Table III that the age and sex distribution of cholera does not materially differ from the age and sex proportions of the population.

IMMUNITY AND RISK OF MORTALITY FROM CHOLERA.

Statistics of deaths from cholera after the commencement of inoculation are available for South Arcot district only. This district has contributed 554 cases of cholera in 392,115 protected persons in 1,283 villages out of the total of 1,118 cases in 709,977 protected persons in 2,350 villages in the present investigation. The statistics of this district are, therefore, a sufficiently large and representative sample of the total experience. This is supported by the fact that the ratio of the incidence of cholera in the protected to the incidence in the unprotected population was 1 : 11·4 as compared with 1 : 10·3 for all the villages in the survey.

- There were 1,124 deaths in 2,439 cases of cholera which occurred in the inoculated persons after the first day of inoculations and 8,956 deaths out of 14,015 in the unprotected population. The case fatality rates in these two groups were 46·08 and 63·90 respectively corresponding to a ratio of 1 : ·1·39. The case fatality rates in the inoculated do not seem to be affected to any material extent by the quantum of immunity in the successive days after inoculation. This may be seen from Table IV :—

TABLE IV.

Case fatality rates in cholera on successive days following inoculation.

Day after inoculation.	Number of cases.	Number of deaths.	Case fatality rate.
1st day	402	188	46·8
2nd day	414	208	50·2
3rd day	406	197	48·5
4th day	298	125	42·0
5th day	224	111	49·6
6th day	141	66	46·8
7th day	90	41	45·1
8th day	62	25	40·3
9th day	38	20	52·6
10th day	30	9	30·0
11th day and above ...	334	134	40·1
TOTAL ...	2,439	1,124	46·08

These figures show that while the protected are about 11 times less liable to contract cholera, their chances of recovery when they get the disease are only about 25 per cent more than the uninoculated.

It is a recognized phenomenon in immunity that adequate levels of anti-bacterial and anti-endotoxic response are obtained only after repeated stimuli with a suitably potent antigen. It may be recalled that, in the present study, the vaccine was administered in a single dose in the greater proportion of instances. Probably, the immunity induced by a single dose of the vaccine, while adequate in the greater majority of cases to arrest the onset of symptoms, breaks down in the presence of massive infection, and, in such instances, the inoculated individual is only at a slightly better advantage regarding recovery than an uninoculated individual getting the disease. Further investigation is necessary to throw light on this question.

DEVELOPMENT OF IMMUNITY.

The time taken for the development of immunity may be determined from the comparative incidence of cholera in an inoculated population at risk on the days following the inoculation. Particulars of the cases of cholera occurring from the first day after inoculation in 1,181,724 inoculated persons in the 2,350 villages in the survey and their statistical significance are given in Table V :—

TABLE V.

Incidence of cholera on successive days following inoculation.

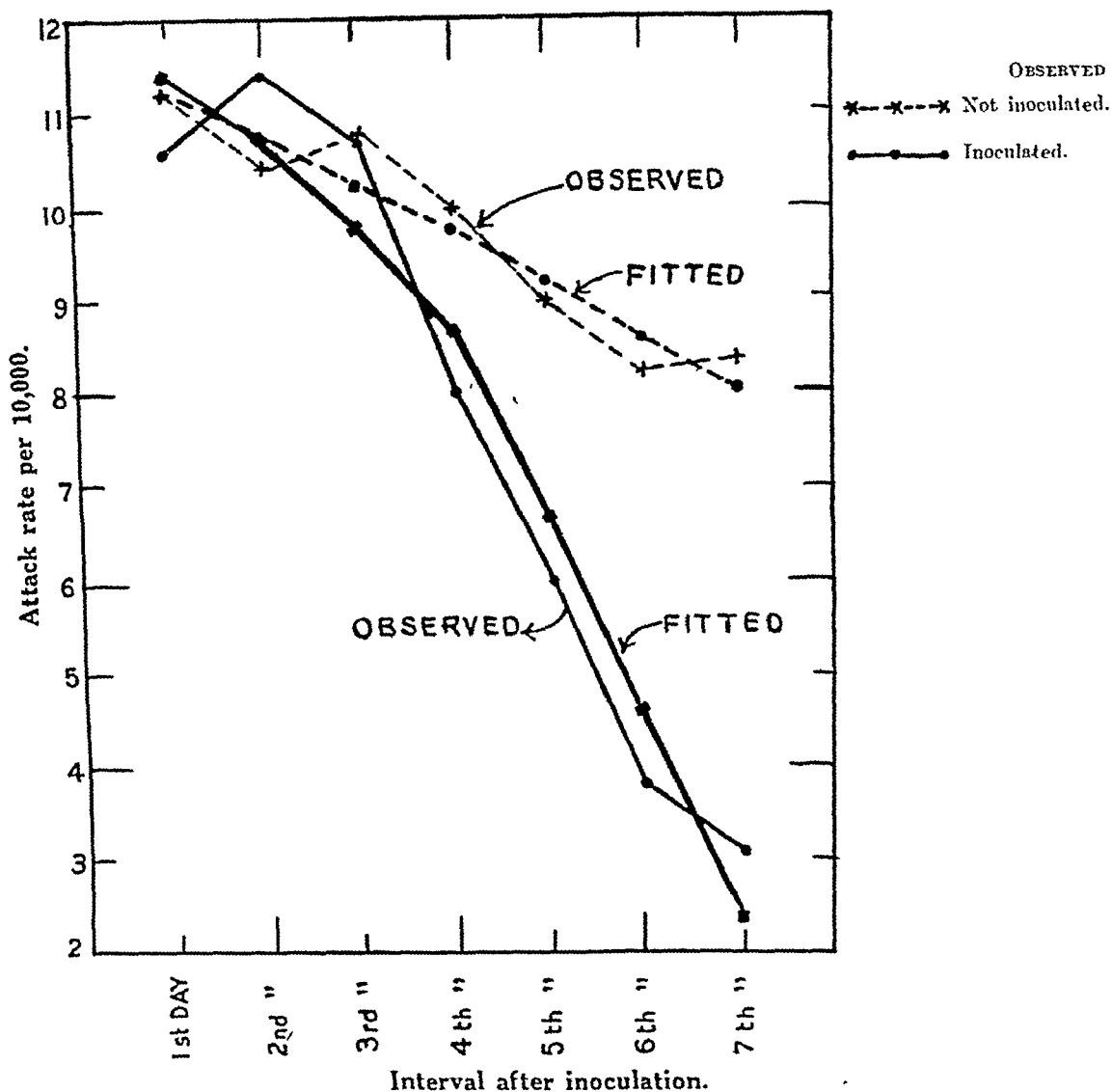| Interval in days after inoculation.	Inoculated population at risk.	Number of attacks in inoculated.	Rate per 10,000.	λ^2	P	REMARKS.
1st day ...	1,181,354	844	7·14			
2nd day ...	1,180,510	891	7·57	1·47	0·23	} Not significant.
3rd day ...	1,179,616	796	6·75	5·62	0·02	
4th day ...	1,178,820	623	5·28	21·01	<0·0016	
5th day ...	1,178,197	474	4·02	20·19	<0·0016	
6th day ...	1,177,723	304	2·58	37·07	<0·0016	} Significant.
7th day ...	1,177,419	204	1·73	19·69	<0·0016	
8th day ...	1,177,215	125	1·06	18·97	<0·0016	
9th day ...	1,177,090	64	0·54	19·69	<0·0016	
10th day ...	1,177,026	66	0·56	0·03	0·86	Not significant.

With reference to the data presented in Table V, it may be argued that in epidemics of cholera, which are generally of short duration, the progressive decrease noticed in the incidence rates from the fourth day after inoculation onwards may not be due to the immunity conferred by inoculation but to the natural decline of the epidemic. It was decided, therefore, to analyse the figures relating only to those persons who were inoculated within the first 24 hours after the health staff arrived in the affected villages for the first time following notification of the occurrence of cholera. The figures for persons inoculated at this time as compared with the uninoculated population are analysed in Table VI. As the Health Staff visited villages for inoculation purposes only after they had received intimation of the occurrence of cholera, it is obvious that a proportion of those inoculated had already been exposed to the risk of infection during the early phases of the epidemic.

TABLE VI.

Incidence of cholera in persons inoculated during the early phases of epidemic.

Interval after inoculation.	INOCULATED.			UNINOCULATED.			χ^2	P	REMARKS.
	Population at risk.	Attacks.	Rate per 10,000.	Population at risk.	Attacks.	Rate per 10,000.			
1st day ...	412,255	438	10·63	1,638,212	1,842	11·24			
							1·14	0·29	
2nd day ...	411,817	471	11·43	1,636,370	1,710	10·45			
							3·02	0·08	Not significant.
3rd day ...	411,346	435	10·59	1,634,660	1,738	10·63			
							0·01	0·92	
4th day ...	410,911	328	7·98	1,632,922	1,614	9·88			
							12·49	<0·0016	
5th day ...	410,583	250	6·08	1,631,308	1,445	8·86			
							30·30	0·0016	Significant.
6th day ...	410,333	156	3·80	1,629,863	1,342	8·23			
							87·78	0·0016	
7th day, ...	410,177	127	3·10	1,628,521	1,354	8·31			

It will be seen from Table VI that the decrease in the incidence of cholera in the inoculated group from the fourth day after inoculation onwards was much greater than the decrease in incidence in the corresponding group of uninoculated persons. The Graph clearly shows that the decreased incidence of cholera in the inoculated group was quite independent of the general trend of the epidemic.

GRAPH.—Trend of cholera incidence in inoculated and uninoculated groups.

Days after inoculation.			OBSERVED, RATE PER 10,000.		FITTED, RATE PER 10,000.	
			Uninoculated.	Inoculated.	Uninoculated.	Inoculated.
1st day	...	...	11·24	10·63	11·26	11·39
2nd ,,	...	...	10·45	11·43	10·73	10·68
3rd ,,	...	...	10·63	10·59	10·20	9·65
4th ,,	...	...	9·88	7·98	9·67	8·30
5th ,,	...	...	8·86	6·08	9·13	6·63
6th ,,	...	...	8·23	3·80	8·59	4·63
7th ,,	...	...	8·31	3·10	8·04	2·32

A still clearer picture of the figures in Tables V and VI is obtained by taking 100 as the index number of cases on the first day after inoculation and comparing with it the number of cases on the succeeding days (*vide* Table VII) :—

TABLE VII.

Index of cholera incidence on successive days following inoculation.

Interval after inoculation.	INOCULATED.		Uninoculated.
	Whole population.	First day only.	
1	2	3	4
1st day ...	100	100	100
2nd day ...	106·2	107·7	93·0
3rd day ...	94·5	99·6	94·6
4th day ...	73·9	73·3	87·9
5th day ...	56·0	57·3	78·8
6th day ...	36·1	35·9	73·2
7th day ...	24·2	29·2	73·9
8th day ...	14·8	Not calculated.	Not calculated.
9th day ...	7·5	...	...
10th day ...	7·0	...	...

Although figures in column 3 of Table VII were available only for seven days following inoculation, it may be inferred from close similarity to the corresponding index numbers in column 2 that there would have been a corresponding similarity in the figures relating to 8th, 9th and 10th days had figures for these days been available.

Attempts were made to ascertain whether immunity increased still further after the tenth day of inoculation. Data relating to cholera cases were available for inoculated persons only. Commencing from the 4th day after inoculation

when immunity first manifests itself, the cholera incidence by weeks in all of the 1·18 million inoculated persons is given in Table VIII :—

TABLE VIII.

Cholera incidence by weeks from fourth day after inoculation onwards.

Period after inoculation.	Inoculated population at the beginning of the week.	Cases of cholera.	Rate per 10,000.	x^2	P	REMARKS.
4th–10th· day ...	1,178,820	1,854	15·73			
				689·66	<0·0016	
11th–17th day ...	1,176,966	172	1·46			
				31·89	<0·0016	Significant.
18th–24th day ...	1,176,794	82	0·70			
				14·46	<0·0016	
25th–31st day ...	1,176,712	40	0·34			
				0·73	0·39	Not significant.
32nd–38th day ...	1,176,672	48	0·41			
TOTAL CASES		2,196	...	...	...	...

The number of cholera cases which occurred after 38th day was negligible and these have been omitted from Table VIII.

The following conclusions may be drawn from the data summarized in Tables V to VIII inclusive :—

(a) Cholera vaccine confers little or no protection in the first three days immediately following inoculation.

(b) The protection afforded by inoculation is first manifested on the fourth day and becomes more pronounced on the succeeding days. The reduction in cholera cases is most marked between the 5th and 6th days and the 8th and 9th days and the 24th to 31st days after inoculation. For practical purposes, it may be inferred that after the 8th day of inoculation, a very high degree of protection against cholera is conferred.

DURATION OF IMMUNITY CONFERRED BY ANTI-CHOLERA INOCULATION.

As has been stated above inoculation of appreciable numbers of persons in a community is generally feasible only during the actual prevalence of cholera. Usually, an outbreak of cholera in a village does not last for more than a few weeks, the mean duration in the present epidemic being 19 days. The interval between the inoculations and the termination of the outbreak is, therefore, too short to study the duration of immunity. Fortunately our records provided data relating to villages in which more than one outbreak of cholera occurred at intervals ranging

A still clearer picture of the figures in Tables V and VI is obtained by taking 100 as the index number of cases on the first day after inoculation and comparing with it the number of cases on the succeeding days (*vide* Table VII) :—

TABLE VII.

Index of cholera incidence on successive days following inoculation.

Interval after inoculation.	INOCULATED.		Uninoculated.
	Whole population.	First day only.	
1	2	3	4
1st day ...	100	100	100
2nd day ...	106·2	107·7	93·0
3rd day ...	94·5	99·6	94·6
4th day ...	73·9	73·3	87·9
5th day ...	56·0	57·3	78·8
6th day ...	36·1	35·9	73·2
7th day ...	24·2	29·2	73·9
8th day ...	14·8	Not calculated.	Not calculated.
9th day ...	7·5	...	...
10th day ...	7·0	...	...

Although figures in column 3 of Table VII were available only for seven days following inoculation, it may be inferred from close similarity to the corresponding index numbers in column 2 that there would have been a corresponding similarity in the figures relating to 8th, 9th and 10th days had figures for these days been available.

Attempts were made to ascertain whether immunity increased still further after the tenth day of inoculation. Data relating to cholera cases were available for inoculated persons only. Commencing from the 4th day after inoculation

outbreak and 627 had multiple outbreaks. These have been classified in Table X according to the proportions of the persons inoculated in the community :—

TABLE X.

Relationship between the occurrence of repeated outbreaks of cholera and the percentage of persons inoculated.

Percentage of population inoculated before the second outbreak.	Number of villages with one outbreak.	Number of villages with two or more outbreaks.	Total.	Percentage of total villages having two or more outbreaks of cholera.
Under 10 ...	93	86	179	48·0
10–30 ...	369	223	592	37·7
30–50 ..	442	183	625	29·3
50–70 ...	444	94	538	17·5
70 and more ...	375	41	416	9·9
TOTAL ...	1,723	627	2,350	...

It will be seen from Table X that, if less than ten per cent of the population is inoculated, the chances for the occurrence of single or multiple outbreaks are almost equal. The chances of the occurrence of multiple outbreaks decrease progressively as the proportion of persons inoculated in the first outbreak increases. The classification of the villages has been made without reference to the sizes of the populations in the several groups ; it may be that, in some groups, a larger number of villages with small populations in whom mass inoculation could be done with comparative ease have been included. From the general trend of the figures, however, it would seem reasonable to infer that the reduction in the number of villages experiencing multiple outbreaks when an increasing proportion of the population is inoculated at the first outbreak is the result of herd immunity. It would appear that, for administrative purposes, inoculation of at least 50 per cent of the population at risk would markedly reduce the chances of second outbreaks.

DISCUSSION.

A statistical evaluation of anti-cholera inoculation as a personal prophylactic against cholera has been given and certain conclusions have been drawn as to its efficacy in the prevention and control of cholera epidemics. The authors are fully aware that a field study of this magnitude in rural areas in India is subject to certain limitations. In the first place, the investigation was not planned as an experiment on the pre-inoculation of a community before the occurrence of an expected epidemic. Data virtually equivalent to such a planned pre-inoculation

experiment were, however, obtained from the present investigation as, in the villages which had two or more outbreaks of cholera, the inoculations done in the first outbreak served as anticipatory inoculations with respect to the second and subsequent outbreaks. Secondly, the reporting agency being comprised chiefly of non-medical personnel, the diagnosis of the cases that were notified as cholera was necessarily based on clinical manifestations without confirmation by bacteriological examination in all cases. Village Headmen being generally conversant with the symptoms of cholera which is of periodical occurrence, the diagnosis can generally be relied on as correct : such errors of omission and commission as must inevitably have occurred are unlikely to be of importance by reason of the large number of cases in the survey, and it is extremely improbable that the significance of the results obtained were materially altered on this account. Thirdly, in order to eliminate inaccuracies in the conduct of the field surveys, in the scrutiny of the records, and in the preparation of the cards by the very large field staff employed, all reasonable precautions by checks and test checks were adopted : as a further safeguard, an independent observer was deputed to check the accuracy of the basic data and he found that such errors as were detected were of a minor nature and inconsequential.

The present study gives an overall picture of the benefits of anti-cholera inoculation in a cross section of the *general* population in rural areas affected by cholera. The conditions prevailing in rural areas as regards water-supplies and environmental and personal hygiene of the inhabitants are such that practically every individual in a given community may be regarded as being at risk, although the degree of risk would undoubtedly be greater in some individuals than in others. When this is kept in mind the great difficulty of selecting groups or communities who could be regarded as being at equal risk will be appreciated. The village or hamlet was adopted as the unit of population for the main statistical investigation. At a later date a re-assessment of the results of this study was made using a smaller unit of population in whom living conditions were considered to be the most uniform that could be obtained. For this purpose the *cheri* (the area inhabited by the depressed classes in a village) was selected as the unit. The results of a special study of the data relating to 63 *cheris* in South Arcot district are given in the following paper by Chandra Sekar (1947, this issue pp. 153–176). The degree of protection afforded by anti-cholera inoculation on the basis of Dr. Chandra Sekar's analysis, though of statistical significance, was appreciably lower than that obtained by our analysis. It must, however, be pointed out that, though the *cheri* population may possess more uniform characters, it must be regarded as an adversely selected population by reason of the extremely low standards of personal and environmental hygiene prevailing in the *cheri*. As even under these exacting conditions of test statistically significant results have been obtained, the efficacy of anti-cholera inoculation may be regarded as established beyond doubt.

SUMMARY AND CONCLUSION.

1. Some 12·48 million persons were inoculated during a widespread and severe epidemic of cholera in the Madras Province in 1942-43.

2. An investigation was carried out on the experience of 1·18 million inoculated persons in 2,350 villages in 11 districts to assess the value of cholera vaccine as

a personal prophylactic and as a measure of prevention and control of cholera epidemics.

3. The vaccine used contained both Inaba and Ogawa sub-types of *V. cholerœ* and was administered in a single dose.

4. There were 1,118 cases of cholera amongst 709,977 protected persons in the inoculated population and 34,336 cases of cholera in 2,119.568 uninoculated persons. The case incidence rates in these two groups of population were 1·57 and 16·20 respectively per 1,000 representing a ratio of 1 : 10·3.

5. Two or more outbreaks of cholera occurred in 627 out of 2,350 villages in the survey. In these villages, the uninoculated population in the second and later outbreaks was 541,808 and the 'protected' population was 281,484 all of whom had been inoculated during the first outbreak. In the second and subsequent outbreaks 6,580 cases of cholera occurred in the uninoculated group and 241 in the 'protected' group. The incidence rates per 1,000 of the respective population groups were 12·14 and 0·86, that is, the incidence in the uninoculated was 14·2 times greater than in the protected group.

6. The results, summarized in paras 4 and 5 above, appear to provide proof that inoculation affords a definite degree of protection against an attack of cholera.

7. In South Arcot district which contributed over 50 per cent of the statistical material in the present inquiry, there were 2,439 cases and 1,124 deaths in the inoculated group, and 14,015 cases and 8,956 in the uninoculated group. The case fatality rates in the two groups are 46·08 and 63·90 per cent respectively; the proportion being 1 : 1·39. There is no significant difference in the fatality rates among the cases occurring in the successive days after inoculations.

8. According to the International Sanitary Convention, anti-cholera inoculation confers effective immunity six days after the date of inoculation. Judged by the incidence of cholera in the inoculated persons in the present investigation, immunity first manifests itself on the fourth day after inoculation and reaches an effective level after the eighth day.

9. Evidence is presented that the immunity conferred by anti-cholera inoculation lasts for a minimum period of six months and probably remains effective up to 12 months.

10. Herd immunity seems to play an important part in preventing multiple outbreaks in a locality during an epidemic. If, during the first outbreak, 50 per cent or more of the population at risk is inoculated, the chances of subsequent outbreaks are greatly reduced.

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STATISTICAL ASSESSMENT OF THE EFFICACY OF ANTI-CHOLERA INOCULATION FROM THE DATA OF 63 *CHERIS* IN SOUTH ARCOT DISTRICT.

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IN the preceding paper by Adiseshan, Pandit and Venkatraman (1947, this issue pp. 131–152) the value of anti-cholera inoculation as a personal prophylactic against cholera and its efficacy in the prevention and control of cholera epidemics have been discussed. The conclusions arrived at are based on an analysis of the field data using a village or hamlet as the unit to define the 'population at risk'. One important consideration in the statistical assessment of the efficacy of inoculation is that the population at risk should, as far as possible, be so chosen that it would be reasonable to consider every individual in it as being exposed to the same amount of risk. In South Indian villages, characteristic differences are found in the environmental and socio-economic conditions in which different groups of the population live. How far such differences would introduce variations in the risk run by the different groups and thus affect the use of a village or hamlet as the unit to define the 'population at risk' was a matter of interest. It was, therefore, suggested that the data originally collected might be submitted to a fresh analysis using a unit smaller in area than a village or hamlet.

The *cheri* was chosen as the new unit. It is usually a compact block inhabited by the 'depressed classes' among the village population. Socially the inhabitants of a *cheri* form a homogeneous group. As they live close together, often use the same source of water-supply and exhibit great similarity in respect of food and other habits, it would seem reasonable to assume that, when cholera breaks out in a *cheri*, the entire population of it would be equally exposed to the risk of infection. For purposes of statistical analysis, therefore, the *cheri* was considered to be a suitable unit to define the population at risk. An advantage in using

this unit was the comparative ease with which information relating to the *cheri* population could be extracted from the village cholera records and inoculation lists.

Figures for the population of the different *cheris* at the time of the 1942-43 cholera epidemic were not readily available and had to be re-constructed by house-to-house visits and by making allowance for such changes in the population as had occurred after the subsidence of the epidemic. Because of the extent of field work involved, it was not possible to extend the inquiry to all the *cheris* included in the original investigation. Attention was confined to one district, South Arcot, which had provided more than half the data for the main investigation. Even in South Arcot, only those *cheris* which had experienced two or more outbreaks during the 1942-43 cholera epidemic were investigated in the present inquiry, as the purpose in view was to determine the effect of inoculation on the incidence of the disease in a fully immunized population. From the report of the main inquiry it will be seen that a second outbreak has been defined as one which took place at least 30 days after the first, so that those who were inoculated during the first outbreak would have attained full protection before exposure to infection at the second and subsequent outbreaks. In view of these limitations the number of *cheris* included in the present study was reduced to 63. The data relating to these *cheris* are discussed in this paper from the point of view of the effect of inoculation on the attack and case-fatality rates of cholera.

The definitions adopted in the main investigation have been followed in this paper.

ASSESSMENT OF THE VALUE OF ANTI-CHOLERA INOCULATION IN PREVENTING DISEASE.

Sixty out of the sixty-three *cheris* included in this investigation suffered only two outbreaks of cholera during the 1942-43 epidemic. For these *cheris*, the number of cholera cases in the second outbreak and the population at risk have been presented in Table I under the three headings : (A) uninoculated, (B) inoculated within 6 days prior to the second outbreak and during the second outbreak, and (C) inoculated during the first outbreak or more than 6 days prior to the second outbreak (anticipatory inoculation). The population figures for group A were obtained by deducting from the population at risk the numbers included in groups B and C. In the remaining three *cheris*, the second outbreak was followed by another after an interval of 30 days. For these *cheris* the data for the last two outbreaks were dealt with separately and the figures for each outbreak have also been shown in Table I under the three groups A, B and C. For the last outbreak the inoculations conducted in the first and second outbreaks provided the figure for the population in group C.

The population inoculated within six days prior to the second outbreak or during the second outbreak gave rise to some difficulties in analysis as, for a part of the second outbreak, it was ' protected ' and for the rest ' unprotected '. Hence

TABLE I.

Showing the number of attacks and deaths from cholera during the second outbreak and the population exposed to risk classified into ' inoculated ' and ' not inoculated ' in each of the 63 cheris.

Village Number.	Name of village.	Attacks.			Deaths.			Population at risk.			POPULATION AT RISK (ADJUSTED).		ATTACK RATE PER 1,000.	
		A*.	B†.	C‡.	A.	B.	C.	A.	B.	C.	Uninoculated.	With anticipatory inoculation.	Uninoculated.	Inoculated.
1	Vellapakkam	3	..	2	1	..	..	518	..	148	548	148	5·5	13·5
2	Cotteri	5	..	1	3	..	..	220	..	71	220	71	22·7	14·1
3	Patharikuppam	1	..	..	1	..	..	200	..	24	200	24	5·0	00·0
4	Alagianatham	4	..	1	3	..	..	235	..	160	235	160	17·0	6·2
5	Maedaiyur	5	..	1	2	..	..	179	..	115	179	115	27·9	8·7
6	Solakkur	1	..	..	..	..	..	43	..	30	43	30	23·3	0·0
7	Lalpuram	4	..	..	3	..	..	123	16	39	132	38	30·3	0·0
8	Nanjalur	1	..	..	1	..	..	81	..	26	81	26	12·3	0·0
9	Thillainayagapuram	6	..	6	3	..	5	200	..	159	200	159	30·0	37·7
10	Poolamedu	1	..	..	1	..	..	176	..	88	176	88	5·7	00·0
11	C. Vakkaramari	1	..	..	3	..	..	95	..	144	95	144	42·1	00·0
12	Kilchavadi	6	..	..	5	..	..	169	81	35	229	31	26·2	00·0
13	Keerapalayam	1	..	1	..	..	1	97	2	1	Discarded		Discarded	
14	Vittalapuram	13	..	1	10	..	..	237	81	61	275	51	47·3	19·6
15	Deovanur	1	..	1	..	..	..	179	..	140	179	140	5·6	7·1
16	Bramadesam	9	..	..	9	..	..	587	122	25	659	14	13·7	0·0
17	Munnur	4	..	1	2	..	1	725	..	90	725	90	5·5	11·1
18	Kapoor	2	..	..	2	..	..	681	254	16	925	15	2·2	0·0
19	Panampet	..	..	1	..	..	1	101	..	389	101	389	0·0	2·6
20	Anangur	1	..	..	1	..	..	98	..	429	98	429	10·2	0·0
21	Maragathapuram	1	..	1	1	..	1	155	..	311	155	311	6·5	3·2
22	Reddikuppam	1	..	1	1	..	1	86	..	94	86	94	11·6	10·6

* A—Uninoculated.
† B—Inoculated within 6 days prior to second outbreak and during the second outbreak.
‡ C—With anticipatory inoculation, i.e. inoculated during the first outbreak or more than 6 days prior to second outbreak.

TABLE I—*contd.*

Village Number.	Name of village.	Attacks.			Deaths.			Population at risk.			POPULATION AT RISK (ADJUSTED).		ATTACK RATE PER 1,000.	
		A*.	B†.	C‡.	A.	B.	C.	A.	B.	C.	Uninoculated.	With anticipatory inoculation.	Uninoculated.	Inoculated.
23	Chittalampattu ..	8	..	..	4	..	..	67	25	227	85	225	94·1	0·0
24	V. Agaram ..	35	4	4	20	2	2	319	121	327	374	312	93·6	12·8
25	Kiltayanur ..	5	..	..	4	..	..	273	..	146	273	146	18·3	0·0
26	Deviagaram ..	4	..	1	3	..	1	252	127	295	294	274	13·6	3·6
27	Kangianur ..	7	..	..	6	..	..	29	152	174	82	161	85·4	0·0
28	Mananpoondi ..	1	..	..	..	..	..	127	..	101	127	101	7·9	0·0
29	Edaiyur ..	10	..	..	3	..	..	200	188	30	316	14	31·6	0·0
30	Tiruvamur (A) ..	..	..	1	..	..	1	202	..	80	202	80	00·0	12·5
31	Tiruvamur (B) ..	4	..	..	3	..	..	61	..	19	61	19	65·6	0·0
32	Pulavanur (A) ..	5	..	..	5	..	..	354	..	33	354	33	14·1	0·0
33	Pulavanur (B) ..	2	..	..	2	..	..	44	7	2	Discarded		Discarded	
34	Kudithangi ..	3	..	..	1	..	..	779	..	236	779	236	3·9	0·0
35	Ezumadu ..	1	..	..	1	..	..	275	..	200	275	200	3·6	0·0
36	Elandampattu (A)	1	..	1	..	..	1	97	..	157	97	157	10·3	6·4
37	Elandampattu (B)	14	..	1	10	..	1	92	..	33	92	33	152·2	30·3
38	Chittarasur ..	9	..	1	6	..	1	155	131	209	256	203	35·2	4·9
39	Ulundiampatti ..	..	..	1	..	..	1	66	..	80	66	80	00·0	12·5
40	Pulichapallam ..	3	..	..	1	..	..	487	159	225	597	215	5·0	0·0
41	C. Arasur ..	3	..	..	3	..	..	124	..	80	124	80	24·2	0·0
42	Venniyur ..	1	..	..	1	..	..	93	..	60	93	60	10·8	0·0
43	TE. Madapuram	1	..	..	1	..	..	88	..	112	88	112	11·4	0·0
44	TE. Manalur ..	10	..	9	7	..	8	122	31	201	141	194	70·9	46·4
45	Vilagam ..	1	..	..	1	..	..	251	..	129	251	129	4·0	0·0
46	Sri Nedunjeri ..	1	..	..	1	..	..	207	197	54	245	16	4·1	0·0
47	Nagarapadi ..	1	..	..	1	..	..	179	..	100	179	100	5·6	0·0

* A—Uninoculated.
† B—Inoculated within 6 days prior to second outbreak and during the second outbreak.
‡ C—With anticipatory inoculation, i.e. inoculated during the first outbreak or more than 6 days prior to second outbreak.

TABLE I—concld.

Village Number	Name of village	Attacks			Deaths			Population at risk			Population at risk (adjusted)		Attack rate per 1,000	
		A*	B†	C‡	A	B	C	A	B	C	Uninoculated	With anticipatory inoculation	Uninoculated	Inoculated
48	C. Sathamangalam—													
	2nd outbreak	1	..	1	1	..	1	358	..	212	358	212	2·8	4·7
	3rd outbreak	5	..	1	2	..	1	357	..	211	357	211	14·0	4·7
49	Vazakollai	..	..	1	..	..	1	106	..	192	106	192	00·0	5·2
50	Kathalai—													
	2nd outbreak	1	..	1	1	..	..	90	..	46	90	46	11·1	21·7
	3rd outbreak	2	..	1	1	..	1	89	..	46	89	46	22·5	21·7
51	Bhutharayampettai	7	..	..	5	..	..	313	74	97	364	92	19·2	00·0
52	Melmanagudi	2	..	..	..	..	1	174	37	3	Discarded		Discarded	
53	Sathapadi	1	..	..	1	..	..	134	..	88	134	88	7·5	00·0
54	Melamungalidy	1	..	..	1	..	..	255	..	105	255	105	3·9	00·0
55	Kuriyamangalam	..	..	1	..	..	1	237	..	159	237	159	00·0	6·3
56	Karamodu	3	1	..	3	1	..	37	18	21	73	17	41·1	0·0
57	Uluthur—													
	2nd outbreak	1	..	1	1	..	..	451	4	203	451	203	2·2	1·9
	3rd outbreak	3	..	2	2	..	1	239	211	207	383	183	7·8	10·9
58	Poovalai	9	..	4	5	..	1	371	1	257	371	257	24·3	15·6
59	Pinnathoor	2	..	..	2	..	..	358	..	70	358	70	5·6	0·0
60	U. Mangalam	..	..	2	..	..	1	207	..	73	207	73	0·0	27·4
61	Kumaramangalam	..	..	1	..	..	..	125	..	207	125	207	0·0	4·8
62	Sathyam	1	..	1	1	..	..	307	4	78	307	78	3·3	12·8
63	Kovilnur	13	3	2	10	2	1	255	315	193	335	138	38·8	14·5
	Total (excluding 13, 33, 52).	251	8	55	173	5	35	..	..					
	Total (all classes)	256	8	56	173	5	35	..	..		15,592	8,124	16·1	6·8

* A—Uninoculated.
† B—Inoculated within 6 days prior to second outbreak and during the second outbreak.
‡ C—With anticipatory inoculation, i.e. inoculated during the first outbreak or more than 6 days prior to second outbreak.

it was decided to omit the data relating to this population after inoculation and to take into consideration only the risk run by it and its cholera experience before inoculation. The risk to which an individual was exposed was calculated on the basis of the ratio of the length of exposure before inoculation to the total duration of the second outbreak. A small proportion of the persons inoculated during the second outbreak or six days prior to the second outbreak had also been inoculated during the first outbreak. Their risk and cholera experience were allocated to the group of individuals who received anticipatory inoculation. The final figures for the population at risk amongst those uninoculated and those who received anticipatory inoculation are shown in Table I. These two sets of population will be referred to hereafter as 'not inoculated' and 'inoculated'.

Some of the persons included in the 'not inoculated' group might have developed immunity as a result of previous attacks of cholera or of sub-clinical infections. No attempt was, however, made to take this into account in assessing the value of anti-cholera inoculation.

In each of 61 among the 63 *cheris* included in this investigation the last case in the final outbreak occurred within five months of the initiation of the inoculation campaign in the *cheri* during the first outbreak. In the remaining two *cheris* this interval was 5 months and 12 days, and 6 months and 1 day, respectively. The assessment made in this paper refers, for all practical purposes, to the protection conferred on the individual during a period of five months after inoculation.

COMPARISON OF ATTACK RATES IN THE 'INOCULATED' AND 'NOT INOCULATED' POPULATIONS.

As a crude assessment of the value of anti-cholera inoculation, the data for all the *cheris* were taken together and the attack rates in the 'inoculated' and 'not inoculated' populations were compared. Out of 15,592 persons who were not inoculated, 251 developed cholera giving an attack rate of 16·1 per 1,000 persons. Of 8,124 inoculated persons 55 suffered from cholera and the attack rate in the 'inoculated' population was 6·8 per 1,000 persons. To test whether the difference in the attack rates in the 'inoculated' and the 'not inoculated' populations was statistically significant, the χ^2 test was applied to the data presented in the following table :—

	Attacked.	Not attacked.	
Inoculated ...	55	8,069	8,124
Not inoculated ...	251	15,341	15,592
TOTAL ...	306	23,410	23,716

The χ^2 value was 36·5 with 1 degree of freedom and the probability of the χ^2 having a larger value by chance was 1 in 5,000. The indication. therefore, was that inoculation afforded protection from the disease and was useful as a personal prophylactic.

CORRELATION BETWEEN THE ATTACK RATES IN THE 'INOCULATED' AND 'NOT INOCULATED' POPULATIONS

The next step was to study the correlation between the attack rates in the 'inoculated' and 'not inoculated' groups as recorded in this inquiry. Greenwood and Yule (1915) state that upon almost any plausible theory of immunization the attack or fatality rates of both classes 'inoculated' and 'not inoculated' in a series of epidemics of varying severity should be highly correlated and add that this criterion affords a means of coming to a conclusion as to the material accuracy of the data. The study of correlation between the attack rates of these two groups of population will, therefore, throw light on the accuracy of the data. Further, as the severity of an epidemic is measured by the attack rate in the uninoculated, the value of inoculation in epidemics of varying severity will also be brought out.

Two methods were applied (1) due to Greenwood and Yule (*loc. cit.*) and (2) due to Wald (1940). The occurrence in each *cheri* was considered as an epidemic by itself but as the data for each *cheri* were limited, *cheris* with more or less the same attack rate in the 'not inoculated' population were grouped together and the data for the groups were taken for analysis. The *cheris* having attack rates in the uninoculated population in one of the following ranges (158·7 per 1,000 persons to 97·8), (97·8 to 54·8), (54·8 to 28·7), (28·7 to 13·9), (13·9 to 6·21), (6·21 to 2·56) and below 2·56 per 1,000 were grouped together. These intervals were chosen as they provided a suitable frequency distribution of the *cheris* on the basis of the 'normal equivalent deviations*' of the attack rates in the 'not inoculated' population. The intervals as reckoned by normal equivalent deviations corresponded to (—1·0 to —1·3). (—1·3 to —1·6). (—1·6 to —1·9). (—1·9 to —2·2), (—2·2 to —2·5), (—2·5 to —2·8) and below —2·8. The data relating to *cheris* of the same group have been shown in Table II.

The attack rates in the 'inoculated' and 'not inoculated' populations of each group and their corresponding normal equivalent deviations were worked out; these are given in Table III.

The method given by Greenwood and Yule (*loc. cit.*) was applied to the data in Table III. The details of the statistical analysis are shown in the *Appendix*. Considering the data of groups I to VI only. the following relationship was found between k_0 the normal equivalent deviation of the attack rate in the 'not

---

* The normal equivalent deviation x (say) is given by the equation:

$$\text{Attack rate per } 1,000 = \frac{1,000}{\sqrt{2\pi}} \int_{-\infty}^{x} e^{-1/2 x^2} \, dx$$

TABLE II.

Giving the data for the cheris grouped according to the attack rate in the uninoculated population.

Village number.	Attacks A†.	Attacks C‡.	Population at risk A.	Population at risk C.	Village number.	Attacks A.	Attacks C.	Population at risk A.	Population at risk C.	Village number.	Attacks A.	Attacks C.	Population at risk A.	Population at risk C.	Village number.	Attacks A.	Attacks C.	Population at risk A.	Population at risk C.
ATTACK RATE 158·7 TO 97·8 PER 1,000 N.E.D.* —1 TO —1·3.					ATTACK RATE 97·8 TO 54·8 PER 1,000 N.E.D. —1·3 TO —1·6.					ATTACK RATE 54·8 TO 28·7 PER 1,000 N.E.D. —1·6 TO —1·9.					ATTACK RATE 28·7 TO 13·9 PER 1,000 N.E.D. —1·9 TO —2·2.				
Group I.					Group II.					Group III.					Group IV.				
37	14	1	92	33	23	8	0	85	225	7	4	0	132	38	2	5	1	220	71
					24	35	4	374	312	9	6	6	200	159	4	4	1	235	160
					27	7	0	82	161	11	4	0	95	144	5	5	1	179	115
					31	4	0	61	19	14	13	1	275	51	6	1	0	43	30
					44	10	9	141	194	29	10	0	316	14	12	6	0	229	31
										38	9	1	256	203	25	5	0	273	146
										56	3	0	73	17	32	5	0	354	33
										63	13	2	335	138	41	3	0	124	80
															48	5	1	357	211
															50	2	1	89	46
															51	7	0	364	92
															58	9	4	371	257
	14	1	92	33	..	64	13	743	911	..	62	10	1,682	764	..	57	9	2,838	1,272

* Normal equivalent deviation.
† A—Not inoculated.
‡ C—With anticipatory inoculation.

TABLE II—*concld.*

ATTACK RATE 13·9 TO 6·21 PER 1,000 N.E.D. —2·2 TO —2·5.					ATTACK RATE 6·21 TO 3·56 PER 1,000 N.E.D. —2·5 TO —2·8.					ATTACK RATE BELOW 2·56 PER 1,000 N.E.D. BELOW —2·8.				
Village number.	Attacks.		Population at risk.		Village number.	Attacks.		Population at risk.		Village number.	Attacks.		Population at risk.	
	A.	C.	A.	C.		A.	C.	A.	C.		A.	C.	A.	C.
Group V.					**Group VI.**					**Group VII.**				
8	1	0	81	26	1	3	2	548	148	18	2	0	925	15
16	9	0	659	14	3	1	0	200	24	19	0	1	101	389
20	1	0	98	429	10	1	0	176	88	30	0	1	202	80
21	1	1	155	311	15	1	1	179	140	39	0	1	66	80
22	1	1	86	94	17	4	1	725	90	49	0	1	106	192
26	4	1	294	274	34	3	0	779	236	55	0	1	237	159
28	1	0	127	101	35	1	0	275	200	57	1	1	151	203
36	1	1	97	157	40	3	0	597	215	60	0	2	207	73
42	1	0	93	60	45	1	0	251	129	61	0	1	125	207
43	1	0	88	112	46	1	0	245	16					
50	1	1	90	46	47	1	0	179	100					
53	1	0	134	88	48	1	1	358	212					
57	3	2	383	183	54	1	0	255	105					
					59	2	0	358	70					
					62	1	1	307	78					
TOTAL ..	26	7	2,385	1,895	.	25	6	5,432	1,851	..	3	9	2,420	1,398
From above	26	7	2,385	1,895	From above	25	6	5,432	1,851					
19	0	1	101	389	30	0	1	202	80	18	2	0	925	15
39	0	1	66	80	55	0	1	237	159	57	1	1	451	203
49	0	1	106	192	60	0	2	207	73					
					61	0	1	125	207					
Total revised	26	10	2,658	2,556	..	25	11	6,203	2,370	..	3	1	1,376	218

inoculated' population and a_1 the corresponding deviation in the 'inoculated' population

$$k_o = 1\cdot8385a_1 + 2\cdot4789 \qquad\qquad (1)$$

TABLE III.

Showing the 'proportion attacked' and the corresponding normal equivalent deviation in the 'not inoculated' and 'inoculated' populations.

Group.		Proportion attacked.	Normal equivalent deviation.
I.	Not inoculated...	0·1522	−1·03
	Inoculated ...	0·0303	−1·88
II.	Not inoculated...	0·0861	−1·37
	Inoculated ...	0·0143	−2·19
III.	Not inoculated...	0·0369	−1·79
	Inoculated ...	0·0131	−2·22
IV.	Not inoculated...	0·0201	−2·05
	Inoculated ...	0·0071	−2·45
V.	Not inoculated...	0·0109	−2·30
	Inoculated ...	0·0037	−2·68
VI.	Not inoculated...	0·0046	−2·61
	Inoculated ...	0·0032	−2·73
VII.	Not inoculated...	0·0012	−3·04
	Inoculated ...	0·0064	−2·49

The observed values of the normal equivalent deviations and the straight line representing the above equation have been shown diagrammatically in Fig. 1(*a*). Statistical analysis showed that this line fitted the data reasonably well and thus indicated a correlation between the attack rates in the 'inoculated' and 'not inoculated' populations.

The method of Greenwood and Yule was also applied to the data of the seven groups after most of the *cheris* of the original group VII were transferred to groups V and VI for reasons explained in the *Appendix*. The equation to the line obtained in this case was

$$k_o = 2\cdot4281a_1 + 3\cdot7446 \qquad\qquad (2)$$

This line and the observed normal equivalent deviations of the attack rates for the seven groups are shown diagrammatically in Fig. 1(*b*). Statistical analysis showed that the line fitted the data very well. Thus, it was evident that not only significant correlation existed between the attack rates in the 'inoculated' and 'not inoculated' populations but also that the data satisfied a criterion laid down by Greenwood and Yule for their accuracy.

FIG. 1(a).

Showing the correlation between the normal equivalent deviations for the inoculated and uninoculated in groups I to VI.
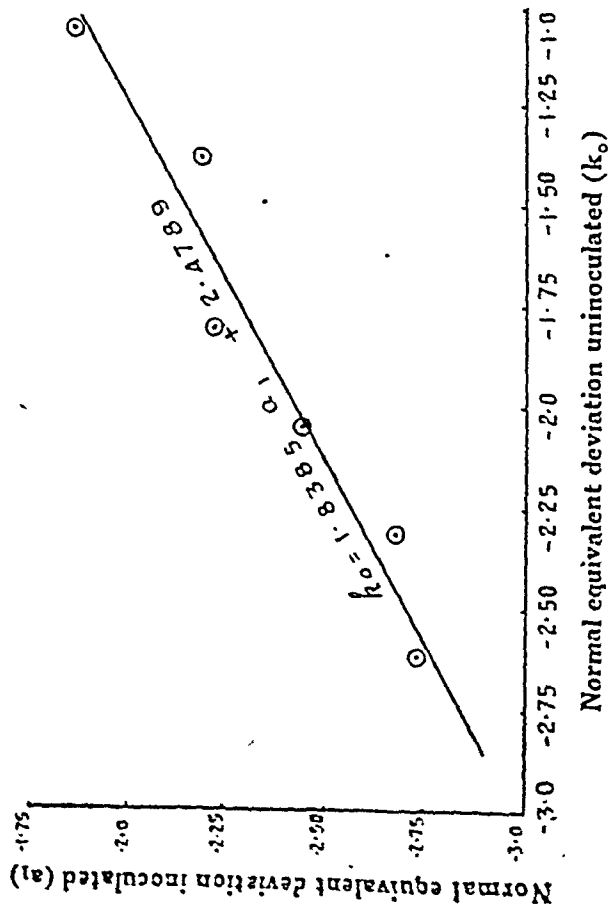
FIG. 1(b).

Showing the correlation between the normal equivalent deviations for the inoculated and uninoculated in groups I to VII (revised).
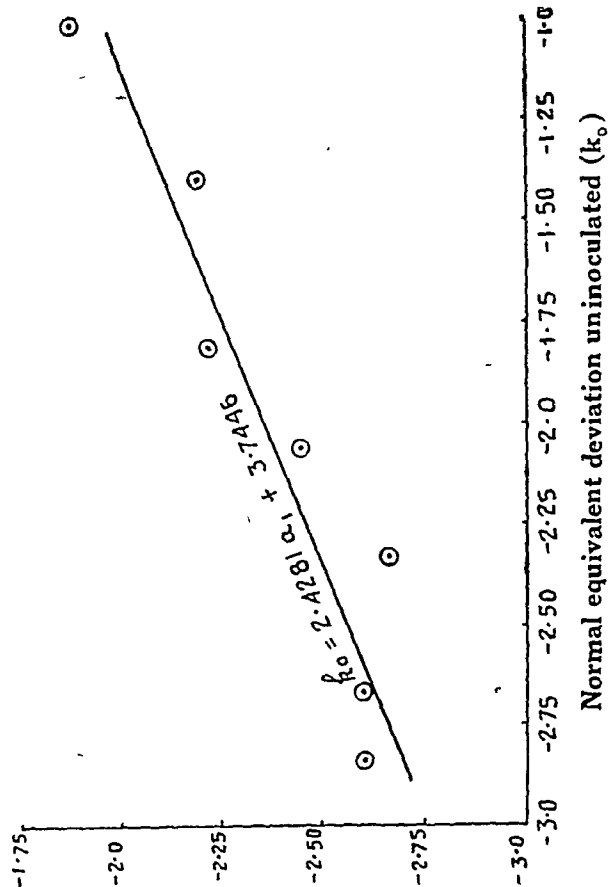
Each of these equations (1) and (2) could be used to represent the variations in resistance of 'not inoculated' and 'inoculated' persons by means of two normal curves. The two pairs of curves corresponding to these equations are shown in Fig. 2. The position of the curve for the 'inoculated' as compared with that for the 'not inoculated' indicates that inoculation increased the average resistance of the group. The greater spread of the curve for the 'inoculated' group shows that inoculation increased the range of variation in resistance between different individuals.

Of the two equations (1) and (2) it is expected that the latter would provide a truer picture of the value of inoculation. The former gives a reasonably accurate picture for outbreaks of higher intensity but exaggerates the value of inoculation in the case of mild outbreaks. This feature is clearly demonstrated when the attack rates to be expected in the 'inoculated' population on the basis of equations (1) and (2) for certain specified attack rates in the 'not inoculated' population are compared. Relevant figures are given in Table IV :—

TABLE IV.

Expected attack rates per 1,000 in the 'inoculated'
population corresponding to a few specified
rates in the 'not inoculated'
population.

Rates per 1,000 not inoculated.	EXPECTED RATES PER 1,000 INOCULATED.	
	Equation (1).	Equation (2).
1	1·2	2·4
5	3·0	4·6
10	4·5	6·2
20	6·9	8·5
30	8·9	10·3
40	10·7	11·8
50	12·4	13·2
75	16·5	16·4
100	20·4	19·2

FIG. 2.—*Distributions of resistance to disease.*

Based on the data of groups I to VI.

Proportion attacked uninoculated.

Proportion attacked inoculated.

Uninoculated.

Inoculated.

R

Scale of resistance.

Based on the data of groups I to VII (revised).

Proportion attacked uninoculated.

Proportion attacked inoculated.

Uninoculated.

The areas under the curves for the 'uninoculated' and 'inoculated' are equal. The proportion of the *population having resistance greater than any arbitrary level* R is given by the proportion of the area of the curve to the right of R. The level of R required to resist disease in any outbreak is obtained by making the proportion of the area of the curve for the 'uninoculated' to the left of R equal to the 'proportion attacked' in the 'uninoculated' population. The corresponding area of the curve for the 'inoculated' will determine the 'proportion attacked' to be expected in the 'inoculated' population.

Inoculated.

R

Scale of resistance.

J. MB

Accepting the results given by equation (2) when the intensity of an epidemic was low and the attack rate amongst the 'not inoculated' was below 5 per 1,000, inoculation hardly offered any protection. For attack rates of 5 to 10 per 1,000 in the 'not inoculated' population the attack rate in the 'inoculated' population was slightly lower. During outbreaks of higher intensity, inoculation offered an increasingly greater degree of protection. Between the ranges of intensity of a cholera epidemic represented by 10 attacks per 1,000 uninoculated to 100 per 1,000, the reduction in attack rate as the result of inoculation was between one-half and four-fifths. The exact relationship between the attack rates in the 'inoculated' and 'not inoculated' populations for outbreaks of varying severity has been shown diagrammatically in Fig. 3 :—

FIG. 3.—*Showing the expected attack rate in the inoculated population for a specified rate in the uninoculated population.*

Attack rate per 1,000 uninoculated.
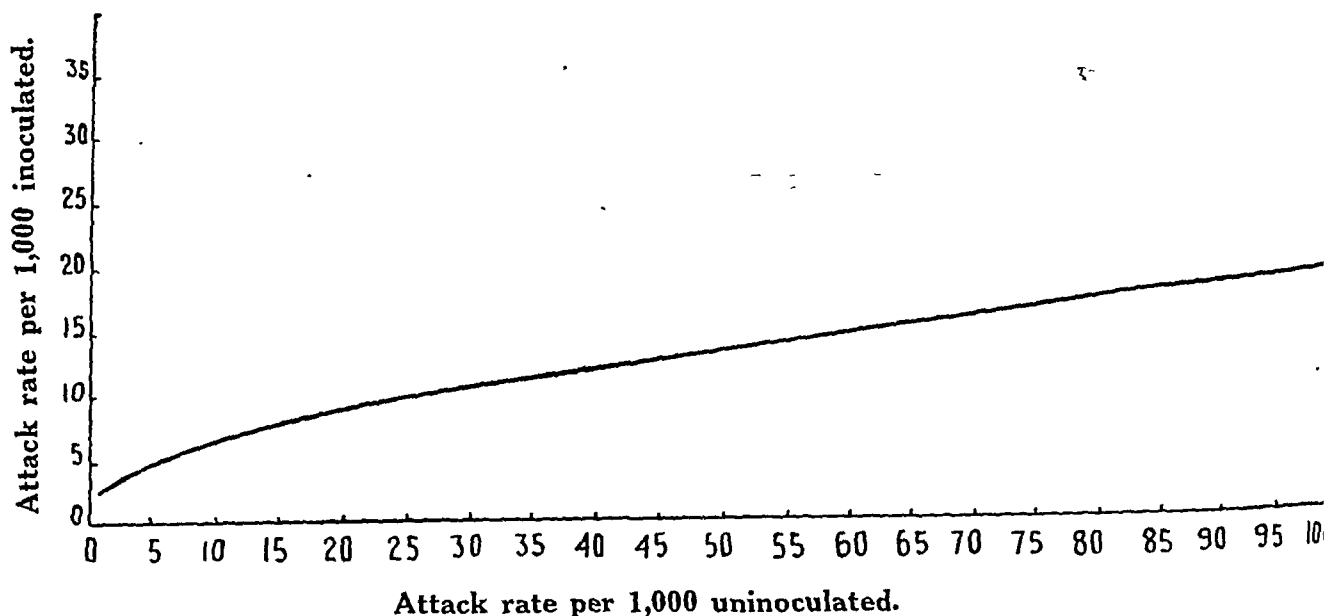
Wald's (*loc. cit.*) method was also used to work out the correlation between the attack rates in the 'inoculated' and 'not inoculated' populations. The details of the statistical analysis are given in the *Appendix*. The following relationship between k_o and a_1 the normal equivalent deviations in the 'not inoculated' and 'inoculated' populations was obtained

$$k_o = 2 \cdot 3185 a_1 + 3 \cdot 4644 \qquad (3)$$

Statistical test showed that this relationship was significantly different from that to be expected if inoculation was of no value. Equation (3) compares well with equation (2) indicating that the conclusions reached by applying the method of Greenwood and Yule are substantiated by the new analysis.

.It should be clearly understood that the extent of protection in the inoculated as assessed above is based on the limited experience of 63 *cheris*; it is possible that a larger experience might have yielded somewhat divergent results. The range of variation that might, in the extreme case, be expected to occur on this account is indicated in the last two columns of Table V. The wide range of such variation in respect of milder outbreaks should be attributed to relatively large errors of sampling.

TABLE V.

Giving the maximum and minimum estimates of the degree of protection against attack given by anti-cholera inoculation.

Rate per 1,000 not inoculated.	RATE PER 1,000 INOCULATED.		RATE IN NOT INOCULATED. RATE IN INOCULATED.	
	Minimum.	Maximum.	Maximum.	Minimum.
1	0·04	6·6	25·0	0·15
5	0·6	8·7	8·3	0·6
10	1·7	10·6	5·9	0·9
20	4·3	15·1	4·7	1·3
30	6·1	21·8	4·9	1·4
40	7·4	30·3	5·4	1·3
50	8·3	39·9	6·0	1·3
75	9·7	68·8	7·7	1·1
100	10·6	102·3	9·4	1·0

The expected maximum rate in the 'inoculated' group is less than the rate in the 'not inoculated' when the intensity of the cholera outbreak rises above the level of 10 cases per 1,000 uninoculated. This should help to strengthen confidence in the conclusions reached previously from the figures in Table IV regarding the efficacy of anti-cholera inoculation.

ASSESSMENT OF THE VALUE OF ANTI-CHOLERA INOCULATION IN REDUCING
CASE FATALITY RATE.

In the 63 *cheris* investigated, 256 attacks were recorded amongst the 'not inoculated' population and of them 173 failed to survive the disease, while of the 56 persons with anticipatory inoculation who were attacked with cholera, 35 failed to survive it. Therefore, the case fatality rates in the 'not inoculated' and 'inoculated' populations were 67·6 per cent and 62·5 per cent respectively. There is little difference between these rates. The χ^2 test when applied to the data in the table below gives a value 0·532 with 1 degree of freedom which is within the 5 per cent significance limit, viz. 3·841 :—

	ATTACKS.		
	Not inoculated.	Inoculated.	Total.
Deaths	173	35	208
Survivals ...	83	21	104
TOTAL ...	256	56	312

It is, therefore, concluded that when a person had an attack of cholera his chance of survival was not influenced by his having been inoculated or not.

DISCUSSION.

The results of statistical assessment of the efficacy of anti-cholera inoculation presented in this paper confirm generally the finding of Adiseshan *et al.* (*loc. cit.*) that anti-cholera inoculation confers significant protection against an attack of cholera though not against death from cholera. The only difference between the two results is in the degree of protection that inoculation affords against attacks of cholera. While Adiseshan, Pandit and Venkatraman find that the attack rate is reduced to 1/10th to 1/14th as a result of inoculation in the present study it is found to be reduced to ½ to 1/5th. This difference may possibly be accounted for by the fact that Adiseshan *et al.* took the larger 'village or hamlet' as the unit of population at risk, while in the present study the smaller and more homogeneous *cheri* was taken as the unit. From the point of view of statistical evaluation of data, the *cheri* is certainly the better unit, and therefore the result obtained on that basis should be more readily acceptable. But judged from other points of view, such as environmental hygiene, immunity status, etc., the *cheri* population is not strictly comparable to the village population. Though the *cheri* forms a part of the village it differs from the rest of the village markedly

due to its lower economic, nutritional and sanitary status. The difference in the degree of protection recorded may be due to such factors. In any case, as even in the *cheri* population anti-cholera inoculation has given significant protection against attacks of cholera, it may safely be concluded that anti-cholera inoculation is not likely to yield less protection than what has been stated in this paper. It is possible that the degree of protection claimed by Adiseshan *et al.* may be obtained in groups of population more favourably placed than the *cheri* population but in population groups exposed to very unfavourable conditions a lower, yet significant, degree of protection as stated in this paper can be expected.

Summary.

1. This paper deals with the assessment of the value of anti-cholera inoculation from the data relating to the second and subsequent outbreaks of cholera in 63 *cheris* in South Arcot district of the Madras Presidency by comparing the attack and case fatality rates among the uninoculated with the corresponding rates among those who were inoculated at least six days prior to the starting of the outbreak dealt with. The latter population should have become fully immunized before this outbreak occurred. Any protection which the uninoculated might have acquired as a result of previous attacks of cholera or of sub-clinical infections was not taken into account in the present inquiry.

In 61 out of the 63 *cheris* investigated the cases of cholera in the second and subsequent outbreaks occurred in the interval one to five months after inoculation. As such the assessment made here refers for all practical purposes to the protection conferred on the individual during a period of five months after inoculation.

2. The attack rate in the 'not inoculated' population was found to be 2·4 times that in the 'inoculated' population. Statistical test indicated that inoculation afforded protection against the disease and was useful as a personal prophylactic.

3. The prophylactic value of anti-cholera inoculation was also assessed by a method suggested by Greenwood and Yule. The results obtained have been presented in Table IV and Fig. 3. Except in very mild outbreaks inoculation was found to be effective. When the attack rate in the 'not inoculated' population was 5 to 10 per 1,000, the attack rate in the 'inoculated' population was slightly lower. During outbreaks of higher intensity, inoculation offered an increasingly greater degree of protection. Between the ranges of intensity of a cholera epidemic represented by 10 attacks per 1,000 uninoculated to 100 per 1,000, the reduction in attack rate, as the result of inoculation, was between one-half and four-fifths.

4. Two normal curves representing the distribution of resistance to disease among the 'uninoculated' and 'inoculated' were worked out and are presented in Fig. 2. The average resistance of the population was increased by inoculation. The range of variation in resistance between inoculated individuals was also greater than the range in the uninoculated group.

5.   There was no significant difference between the case fatality rates amongst those attacked in the ' inoculated ' and ' not inoculated ' populations.

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APPENDIX.

Statistical assessment of the correlation between the attack rates in the 'inoculated' and 'not inoculated' populations.

As explained earlier in the text, the *cheris* were divided into seven groups on the basis of the attack rates in the 'not inoculated' population and for the data of each group the attack rates in the 'inoculated' and 'not inoculated' populations and the corresponding normal equivalent deviations were worked out. These have already been presented in Table III of the text.

In all the groups except the last one, the attack rate in the 'inoculated' population was less than that in the 'not inoculated'. The higher rate amongst the 'inoculated' population in group VII could be anticipated as this group included all the *cheris* in which no case had occurred amongst the uninoculated persons though in each of these *cheris* there was at least one case amongst the inoculated persons. Statistical assessment of the correlation in attack rates is rendered difficult by the data in group VII. An assessment made without taking these data into account will give a fairly accurate picture for higher intensities of outbreak but may exaggerate the value of inoculation in the case of mild outbreaks. On the other hand, if the analysis is made by taking these data in a distinct group, it would unduly prejudice the value of inoculation in mild outbreaks. In what follows, the method of Greenwood and Yule for assessing the correlation between the attack rates in the 'inoculated' and 'not inoculated' populations has been applied to two sets of data. The first consists of the data of groups I to VI only. The second includes those for group VII also but not as a distinct group. The true relationship is expected to be nearer the one given by the second set of data though the results of the first set would also give a fairly accurate picture for higher intensities of outbreak.

Wald's method for working out the correlation between two sets of variables has also been applied to the second set of data and the maximum and minimum limits to the efficacy of inoculation as given by these have been determined.

Method of Greenwood and Yule.—In this method the pair of normal equivalent deviations for the different groups are tabulated and their means M_o and M_1, standard deviations S_o and S_1 (the subscript $_o$ refers to the 'not inoculated') and the coefficient of correlation r are worked out. The relationship between k_o and a_1, the normal equivalent deviations of the attack rates in the 'not inoculated' and 'inoculated' populations is then given by

$$k_o = a_1 \cot \theta + d \qquad (1)$$

$$\text{where } \tan 2\theta = \frac{2rS_oS_1}{S_o^2 - S_1^2} \qquad (2)$$

$$\text{and} \qquad d = M_o - M_1 \cot \theta \qquad (3)$$

Data of groups I to VI.

Applying the method of Greenwood and Yule to the attack rates in groups I to VI, it is found that

$$M_o = -1.86 \qquad M_1 = -2.36$$
$$S_o = 0.536 \qquad S_1 = 0.296$$
$$\text{and } r = 0.973$$
$$\text{Therefore } \tan 2\theta = 1.5449$$
$$\theta = 28° 32.5$$
$$\text{and } \cot \theta = 1.8385$$

The relationship between k_o and a_1 therefore works out to

$$k_o = 1.8385 \, a_1 + 2.4789 \qquad (4)$$

To appreciate the extent to which this relationship fits the data, the observed attack rates in the 'inoculated' and 'not inoculated' populations should be compared with those expected on the basis of this relationship. The expected rates are obtained by, first calculating $k_o{}^1$ and $a_1{}^1$, the expected values of the normal equivalent deviations for the 'not inoculated' and 'inoculated' populations by applying the formulæ:

$$k_o{}^1 = \frac{a_1 \cot \theta + d + k_o \cot^2 \theta}{1 + \cot^2 \theta} \qquad (5)$$
$$\text{and } a_1{}^1 = \frac{k_o{}^1 - d}{\cot^2 \theta} \qquad (6)$$

where k_o and a_1 are the observed normal equivalent deviations, and then converting the normal equivalent deviations to rates.

The proportions of attacks corresponding to the observed and expected values of the normal equivalent deviations are shown in Table A. The difference between the observed and expected values of these proportions and the ratio of the difference to its standard error are also shown in the table. The ratios are generally small signifying that the relationship worked out above gives a fairly good fit. The sum of the squares of these ratios, viz. 2.364, is less than 9.488, the 5 per cent significant limit of χ^2 distribution with 4* degrees of freedom and shows again that the fit is reasonably good.

Data of groups I to VII.

As already mentioned, the exclusion of the data in group VII would result in an exaggeration of the value of inoculation especially in the case of mild outbreaks. To avoid this bias and at the same time to ensure that one group was not unduly weighted, by the inclusion within it of all the *cheris* in which no case was recorded in the 'uninoculated' population, the following method was adopted to deal with the data of this group. For each *cheri* in group VII in which no case was recorded in the 'uninoculated' population the attack rate that would possibly have occurred amongst the 'not inoculated', if a larger number of such persons

---

* It is difficult to give the degrees of freedom precisely. Two constants have been worked out from six pairs of observations. The deviations of each pair from the fitted line are correlated. Hence the test with the degree of freedom chosen can only err by being rather stringent.

TABLE A.

Showing the observed and expected 'proportion attacked' in the 'not inoculated' and 'inoculated' populations.

Group.	Normal Equivalent Deviation.		Proportion Attacked.		Difference in Proportions. Observed-expected.	Standard error of difference.	Difference in proportions / Standard error.	$\left(\dfrac{\text{Difference in proportions}}{\text{Standard error}}\right)^2$
	Observed.	Expected.	Observed.	Expected.				
I. { Not inoculated	−1·03	−1·02	0·1522	0·1539	−0·0017	0·0376	−0·0452	0·0020
{ Inoculated	−1·88	−1·90	0·0303	0·0287	0·0016	0·0291	0·0550	0·0030
II. { Not inoculated	−1·37	−1·41	0·0861	0·0793	0·0068	0·0099	0·6869	0·4718
{ Inoculated	−2·19	−2·12	0·0143	0·0170	−0·0027	0·0043	−0·6279	0·3943
III. { Not inoculated	−1·79	−1·75	0·0369	0·0401	−0·0032	0·0048	−0·6667	0·4445
{ Inoculated	−2·22	−2·30	0·0131	0·0107	0·0024	0·0037	0·6486	0·4207
IV. { Not inoculated	−2·05	−2·04	0·0201	0·0207	−0·0006	0·0027	−0·2222	0·0494
{ Inoculated	−2·45	−2·46	0·0071	0·0069	0·0002	0·0023	0·0870	0·0076
V. { Not inoculated	−2·30	−2·33	0·0109	0·0099	0·0010	0·0020	0·5000	0·2500
{ Inoculated	−2·68	−2·62	0·0037	0·0044	−0·0007	0·0015	−0·4667	0·2178
VI. { Not inoculated	−2·61	−2·59	0·0046	0·0048	−0·0002	0·0009	−0·2222	0·0494
{ Inoculated	−2·73	−2·76	0·0032	0·0029	0·0003	0·0013	0·2308	0·0533
								2·3838

had been exposed to risk, was estimated. The ground for this estimation was that, with this rate, the chance of getting no case amongst the observed 'not inoculated' persons would be $\frac{1}{2}$. On the basis of this estimated attack rate the *cheris* were transferred to the appropriate groups. The application of the procedure resulted in the transference of the observed data of three of the *cheris* in group VII to group V and of four others to group VI. The two *cheris* in which cases had occurred amongst the 'not inoculated' population were left in group VII itself. The attack rates amongst the 'not inoculated' and 'inoculated' populations in the seven groups thus reconstituted and the corresponding normal equivalent deviations are presented in Table B.

Following the method of Greenwood and Yule for estimating the relationship in attack rates it is found that

$$M_o = -2 \cdot 01 \qquad\qquad M_1 = -2 \cdot 37$$
$$S_o = 0 \cdot 614 \qquad\qquad S_1 = 0 \cdot 265$$
$$\text{and} \quad r = 0 \cdot 935$$
$$\text{Therefore, } \tan 2\,\theta = 0 \cdot 9924$$
$$\theta = 22^\circ \ 23'$$
$$\text{and } \cot \theta = 2 \cdot 4281$$

The relationship between k_o and a_1 reduces to

$$k_o = 2 \cdot 4281 \, a_1 + 3 \cdot 7446 \qquad\qquad (7)$$

For the study of the extent to which the line given by equation (7) fits the observed data, the 'proportion of attacked' expected on the basis of this relationship was compared with that observed in the field. The comparative figures are also indicated in Table B. The ratio of the difference between the observed and expected proportions in terms of its standard error is also given in the table. These ratios are not large enough to indicate any significant difference between the observed and the expected proportions. The sum of squares of the ratios works out to $3 \cdot 877$ which is less than $11 \cdot 070$, the 5 per cent significant limit of χ^2 with 5 degrees of freedom. There is, therefore, every reason to believe that equation (7) describes adequately the relationship between the attack rates amongst the 'inoculated' and 'not inoculated' populations.

Wald's method.

This method is not strictly applicable to our data as the latter fail to satisfy the condition of constancy of variance of one set of variables. As this limitation is not likely to be of serious consequence, the method has been applied to the normal equivalent deviations given in Table B above. The deviations of group IV have been excluded and the rest have been divided into two sets, the first comprising the deviations of groups I, II and III and the second of V, VI and VII. The relationship between k_o and a_1, the normal equivalent deviations of the 'not inoculated' and 'inoculated' populations, is found to be

$$k_o = 2 \cdot 3185 \, a_1 + 3 \cdot 4644 \qquad\qquad (8)$$

If inoculation did not produce any effect, the coefficient of a_1 in equation (8) should not be significantly different from unity and the constant term should not be different from zero. The F test for significance gives a value $10 \cdot 4$ which is

TABLE B.

Showing the observed and expected 'proportion attacked' in the 'not inoculated' and 'inoculated' populations.

Group (revised).	Normal Equivalent Deviation. Observed.	Normal Equivalent Deviation. Expected.	Proportion Attacked. Observed.	Proportion Attacked. Expected.	Difference in Proportions. Observed-expected.	Standard error of difference.	Difference in proportions / Standard error.	(Difference in Proportions / Standard error)²
I. { Not inoculated ...	−1·03	−1·00	0·1522	0·1587	−0·0065	0·0381	−0·1706	0·0291
{ Inoculated ...	−1·88	−1·95	0·0303	0·0256	0·0047	0·0275	0·1709	0·0292
II. { Not inoculated ...	−1·37	−1·40	0·0861	0·0908	−0·0053	0·0100	0·5300	0·2809
{ Inoculated ...	−2·19	−2·12	0·0143	0·0170	−0·0027	0·0043	−0·6279	0·3943
III. { Not inoculated ...	−1·79	−1·77	0·0389	0·0384	−0·0015	0·0047	−0·3191	0·1018
{ Inoculated ...	−2·22	−2·27	0·0131	0·0116	0·0015	0·0039	0·3846	0·1479
IV. { Not inoculated ...	−2·05	−2·07	0·0201	0·0192	0·0009	0·0026	0·3462	0·1199
{ Inoculated ...	−2·45	−2·40	0·0071	0·0082	−0·0011	0·0025	−0·4400	0·1936
V. { Not inoculated ...	−2·33	−2·39	0·0098	0·0084	0·0014	0·0018	0·7778	0·6050
{ Inoculated ...	−2·66	−2·52	0·0039	0·0059	−0·0020	0·0015	−1·3333	1·7777
VI. { Not inoculated ...	−2·05	−2·04	0·0040	0·0041	−0·0001	0·0008	−0·1250	0·0156
{ Inoculated ...	−2·60	−2·63	0·0046	0·0043	0·0003	0·0013	0·2308	0·0533
VII. { Not inoculated ...	−2·85	−2·81	0·0022	0·0025	−0·0003	0·0013	−0·2308	0·0533
{ Inoculated ...	−2·60	−2·70	0·0046	0·0035	0·0011	0·0040	0·2750	0·0756
								3·8772

greater than the 5 per cent limit of F, viz. 6·94. It is, therefore, concluded that inoculation did produce some effect and as equation (8) compares well with that obtained by applying the method of Greenwood and Yule, the conclusions reached earlier regarding the efficacy of anti-cholera inoculation may be considered as substantiated.

If

$$k_o = a\, a_1 + \beta \tag{9}$$

be the relation between the normal equivalent deviations, the confidence region for a and β with a chance of 0·95 is given by the ellipse,

$$5\cdot5877\, a^2 + \beta^2 - 4\cdot7166\, a\, \beta - 9\cdot6406\, a + 4\cdot0066\, \beta + 4\cdot1341 = 0 \tag{10}$$

Any pair of values of a and β in the region enclosed by the ellipse can lead to such difference in attack rates in the 'inoculated' and 'not inoculated' populations as was observed in the field. Each pair of values will assess differently the efficacy of anti-cholera inoculation. The maximum and minimum estimates for this efficacy have been given in Table V of the text.

ANTISEPTICS OF THE ACRIDINE SERIES.

Part I.

EFFECT OF VARIOUS SUBSTITUENTS AND LOADING OF THE TERMINAL N—IN THE DIALKYLAMINO ALKYL SIDE CHAIN OF TYPE—NH(CH₂)₄NR₂ AT POSITION 9 IN 3-METHOXY-5-CHLORO-9-AMINO-ACRIDINE.

BY

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CHEMOTHERAPEUTIC investigations of mono- and diamino-acridines (Albert, 1936 ; Rubbo *et al.*. 1941) have shown that simple mono-amino-acridines— especially 9-amino-acridines (I)—are the most active ones. Das Gupta and Gupta (1945) have shown that 5-(diethyl-amino-alkyl)-amino-acridines (II) possess considerable anti-septic activity. Dupre and Robinson (1945) during their study of N-substituted

where R = alkyl radical.

(Ethyl, propyl, butyl, etc.)

I
9-amino- acridine.

II
5-diethyl-amino-alkyl-amino-acridine.
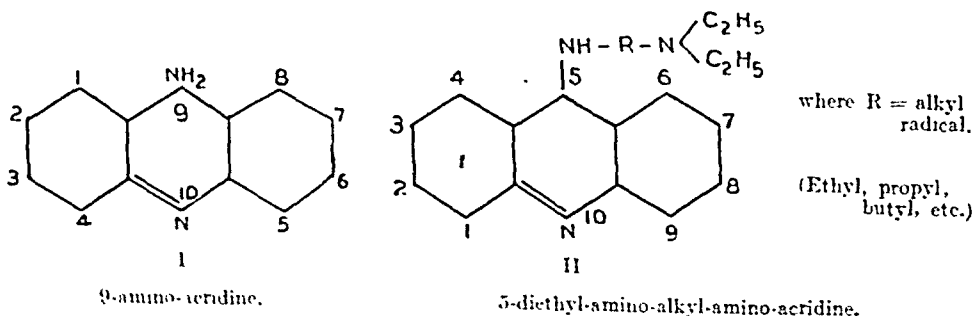
5-amino-acridines found that the parent 5 amino-acridine was as active bacterio-statically as the substituted compounds.   Lawrence *et al.* (1944) have also described the antiseptic action of many common acridine derivatives on Gram-positive and Gram-negative bacteria.   It is also known (Eggerth, 1926) that acridine compounds are not inactivated in the presence of serum and organic matter.

Several basically substituted chloro-methoxy 9-amino-acridines have been synthesized in the Chemistry Department, Government College, Lahore.   Intro-duction of chlorine in the molecule of various phenols is known to confer high antiseptic properties (Klarmann *et al.*, 1929).   The activating effect of chlorine in the phenolic series has prompted us to study the effect of the introduction of chlorine in the acridine series.   Shaw (1928) has shown that the presence of a methoxy group is also desirable, because it increases the permeability of the drug. As these compounds are substituted 9-amino-acridines with a chlorine and a methoxy radicle, we have undertaken to find out if there is any relation between antiseptic activity and chemical constitution.

In this paper, the following derivatives of 3-methoxy-5-chlor-9-amino-acridine (III) have been examined :—
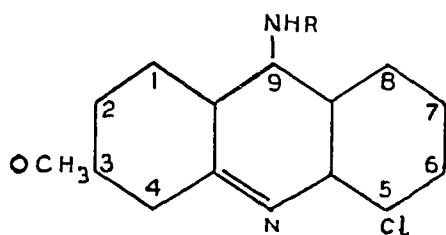
III
3-methoxy-5-chlor-9-amino-acridine.

where R = 1. $-(CH_2)_4\ N\!<\!{}^{C_2H_5}_{C_2H_5}$

2. $-(CH_2)_4-N(C_3H_7)_2$

3. $-(CH_2)_4-N(C_4H_9)_2$

4. $-(CH_2)_4-N(C_5H_{11})_2$

5. $-(CH_2)_4-N\!<\!{}^{CH_2-CH_2}_{CH_2-CH_2}\!>\!CH_2$

6. $-\!\langle\ \rangle\!-SO_2NH_2$

7. $-H$

1.   3-methoxy-5-chlor-9 (σ diethyl-amino-butyl) amino-acridine di-hydro-chloride trihydrate.

2.   3-methoxy-5-chlor-9 (σ di-n-propyl-amino-butyl) amino-acridine di-hydro-chloride dihydrate.

3.   3-methoxy-5-chlor-9 (σ di-n-butyl-amino-butyl) amino-acridine di-hydro-chloride.

4.   3-methoxy-5-chlor-9 (σ di-n-amyl-amino-butyl) amino-acridine di-hydro-chloride.

5. 3-methoxy-5-chlor-9 (σ piperidino-butyl) amino-acridine di-hydrochloride penta-hydrate.

6. 3-methoxy-5-chlor-9 (p'sulphanil-amino-phenyl) amino-acridine hydro-chloride.

7. 3-methoxy-5-chlor-9-amino-acridine.

8. Acriflavine B.P.

9. Quinacrine hydrochloride U.S.P.

Drugs Nos. 8 and 9 were used as standards for comparison of the activities.

TECHNIQUE.

A modification of the method of Lawrence *et al.* (*loc. cit.*) was used. This is outlined below :

1 : 1,000 dilution of each compound was made in simple broth containing 0·1 per cent dextrose. From this dilution, dilutions of 1/2000, 1/4000, 1/8000, 1/16000, 1/32000, 1/64000, 1/128000, 1/256000 were made. The tubes containing the drug dilutions and control broth tubes not containing the drug were autoclaved at 10 lb. for 10 minutes. Each tube of the dilution series was then inoculated with a loopful of a 24-hour broth culture containing one of the test organisms.

The tubes were then incubated at 37°C. and examined for visible growth after 24, 48 and 72 hours. Lack of growth after 24 hours was taken as evidence of bacteriostasis. The first tube which failed to show growth after 72 hours was tested for the presence of live organisms by transferring 3 loopfuls from this tube to a tube of sterile broth not containing the drug. Failure of the growth to appear in the subculture after 24 hours was taken as evidence of bactericidal action on the part of the drug under investigation.

The compounds which were insoluble in water were dissolved in the minimum possible quantity of absolute alcohol and then diluted with broth so as to yield a 1 in 10,000 dilution. This dilution was clear and free from any precipitate. Five c.c. of absolute alcohol were also added to those drugs which were soluble in water so as to create similar conditions in the experiments.

After removal from the autoclave the tubes containing a high concentration of the drug usually showed floccules which settled down to the bottom of the tube in 24 hours. The reason for this flocculation is not known. It did not. however, interfere with the reading of the results.

The following test organisms were used in the experiments :—

    1. *Staphylococcus aureus.*
    2. *Streptococcus hæmolyticus.*
    3. *Bact. coli.*
    4. *Proteus vulgaris.*
    5. *Pseudomonas pyocyaneus.*
    6. *Bact. typhosum.*

The results are summarized in Tables I, II and III.

TABLE I.

Bacteriostatic and bactericidal values in 0·1 per cent glucose broth of the compounds used in the experiment.

Compound number.	Staph. aureus.		Strept. hæmo.		Bact. coli.		Proteus vulgaris.		Pseudo. pyocya.		Bact. typhosum.	
	Bs.	Bc.	Bs.	Bc.	Bs.	Bc.	Bs.	Bc.	Bs.	Bc.	Bs.	Bc.
1	8,000	4,000	16,000	16,000	16,000	8,000	×	×	×	—	—	—
2	32,000	8,000	64,000	32,000	64,000	32,000	2,000	1,000	×	—	—	—
3	8,000	4,000	8,000	4,000	4,000	2,000	×	—	×	—	—	—
4	8,000	4,000	8,000	4,000	4,000	×	×	—	×	—	—	—
5	16,000	8,000	32,000	16,000	32,000	16,000	2,000	—	×	—	—	—
6	8,000	4,000	8,000	2,000	8,000	2,000	8,000	2,000	—	—	8,000	4,000
7	160,000	160,000	160,000	160,000	320,000	320,000	10,000	<10,000	<10,000	<10,000	—	—
8	64,000	64,000	128,000	32,000	256,000	128,000	128,000	64,000	—	—	128,000	32,000
9	8,000	4,000	×	—	×	—	×	—	—	—	×	—

Bs. = denotes the bacteriostatic concentration.
Bc. = denotes the bactericidal dilution.
× = less than 1 in 1,000.
— = not tried.

TABLE II.

Highest dilution of the drug showing bactericidal action in presence of normal ox-serum

Compound number.	Staph. aureus.	Bact. coli.	REMARKS.
1	8,000	16,000	The method used was essentially the same, as described previously—with the exception that no autoclaving was done, and after 72 hours subcultures were made on glucose broth, agar slope—MacConkey plate and MacConkey liquid medium. Absence of growth in the majority of the media was taken as an evidence of bactericidal action on the part of the drug.
2	16,000	32,000	
3	4,000	4,000	
4	4,000	2,000	
5	16,000	32,000	
6	<10,000	<10,000	
7	>320,000	640,000	
8	>160,000	320,000	
9	<10,000	<10,000	

IRRITANT PROPERTIES.

A strong solution of the drug in normal saline was instilled into one eye of the rabbit and in the other eye an equal amount of normal saline was dropped. The colour of the conjunctivæ was observed and compared with that of the control. A fresh charge of 2 to 4 drops of the drug was instilled after 5 minutes if no reaction ensued. Three rabbits were used for each drug and the experiment was repeated to confirm the observation.

TABLE III.

Compound number.	REACTION (COLOUR OF THE CONJUNCTIVÆ) AFTER					Conc. used.	REMARKS.
	5 minutes.	10 minutes.	30 minutes.	24 hours.	Control.		
1	No redness.	No redness.	No redness.	White	White	1 : 250	There were no signs of uneasiness in the animals. Pupils did not contract or dilate. There was no swelling of the eye-lids or the conges- tion of the eye in general.
2	,,	,,	,,	,,	,,	1 : 250	
3	,,	,,	,,	,,	,,	1 : 250	
4	,,	,,	,,	,,	,,	1 : 250	
5	,,	,,	,,	,,	,,	1 : 250	
6	,,	,,	,,	,,	,,	Saturated solution in water.	
7				,,	,,	1 : 10,000	
8	Light yellow.	Pale	White	,,	,,	1 : 500	
9	White	White	,,	,,	,,	1 : 250	

DISCUSSION OF RESULTS.

.   A glance at the tables will reveal the following facts :—

1.   The parent 3-methoxy-5-chlor-9-amino-acridine is the most active compound of the lot.   Its activity of 1 : 320,000 and 1 : 160,000 against *Bact. coli* and *Staph. aureus* is almost 2½ times that of acriflavine which has values of 1 : 128,000 and 1 : 64,000 for the above organisms respectively. It is less active against *Proteus vulgaris* and is almost inactive against *Ps. pyocyaneus*.   It may be remarked that our observation confirms the work of Dupre and Robinson (*loc. cit.*) who also found that unsubstituted 5-amino- (English nomenclature corresponding to 9 amino in our case) acridine was more active than any of the substituted ones.

GRAPH 1.

Showing relation between bacteriostatic values and the increase in R in
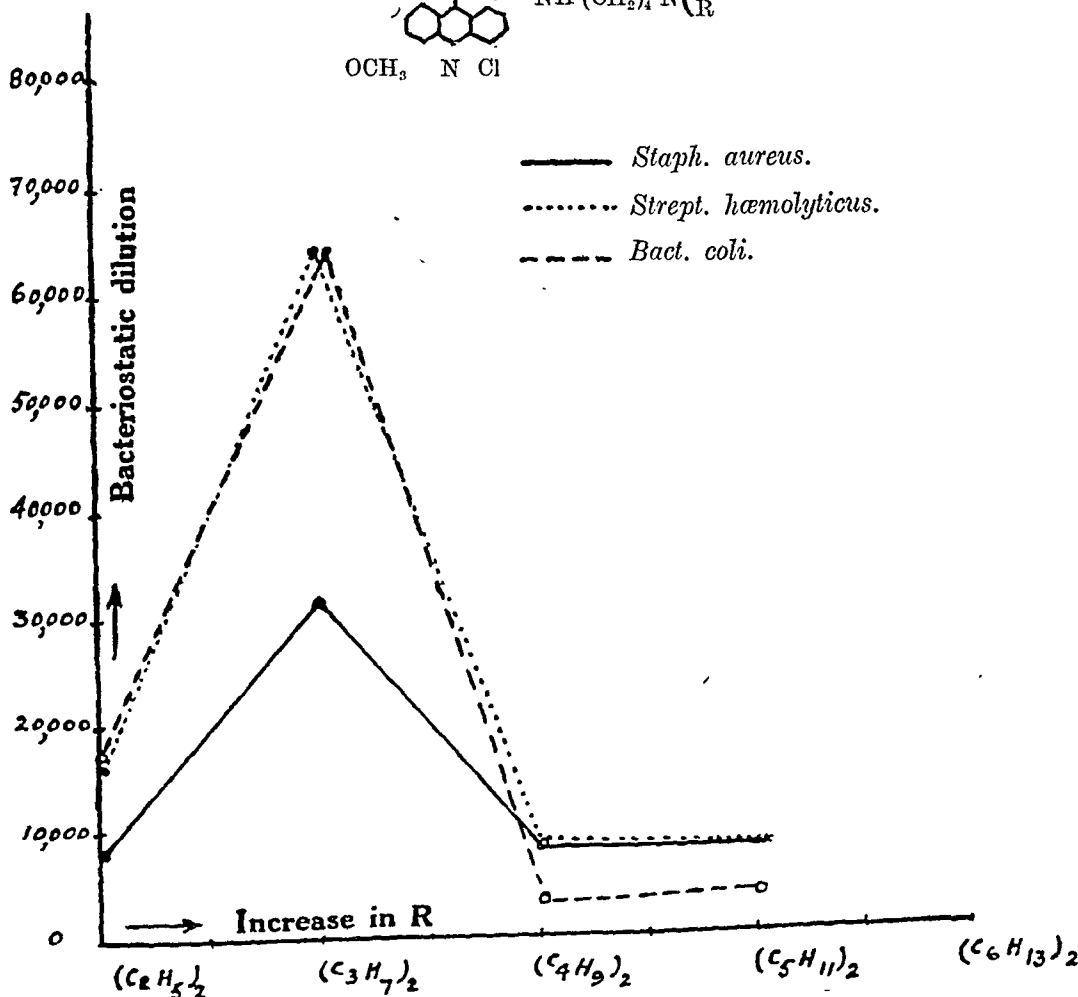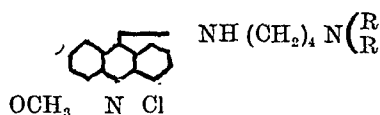
2. The activity rises when R [in $NH(CH_2)_4NR_2$] is changed from ethyl to propyl—then it is followed by a sharp 'fall, when R is butyl, and the curve becomes straight when R reaches amyl (*see* Graph 1). But as the compounds when R is CH_3 and C_6H_{13} and C_7H_{15} were not available, no tentative conclusion can be drawn as regards the relation of antiseptic activity and chemical constitution.

3. When R = piperidine = C_5H_{10}—a closed ring substituent—its activity is almost double that of diethyl compound (C_4H_{10}).

4. Combination of two antiseptic groupings—sulphanilamide and acridine nucleus—does not result in any marked increase in activity except that the compound is uniformly active against all the organisms tested.

GRAPH 2.

Showing the relation between bactericidal dilution and the increase in the value of R in
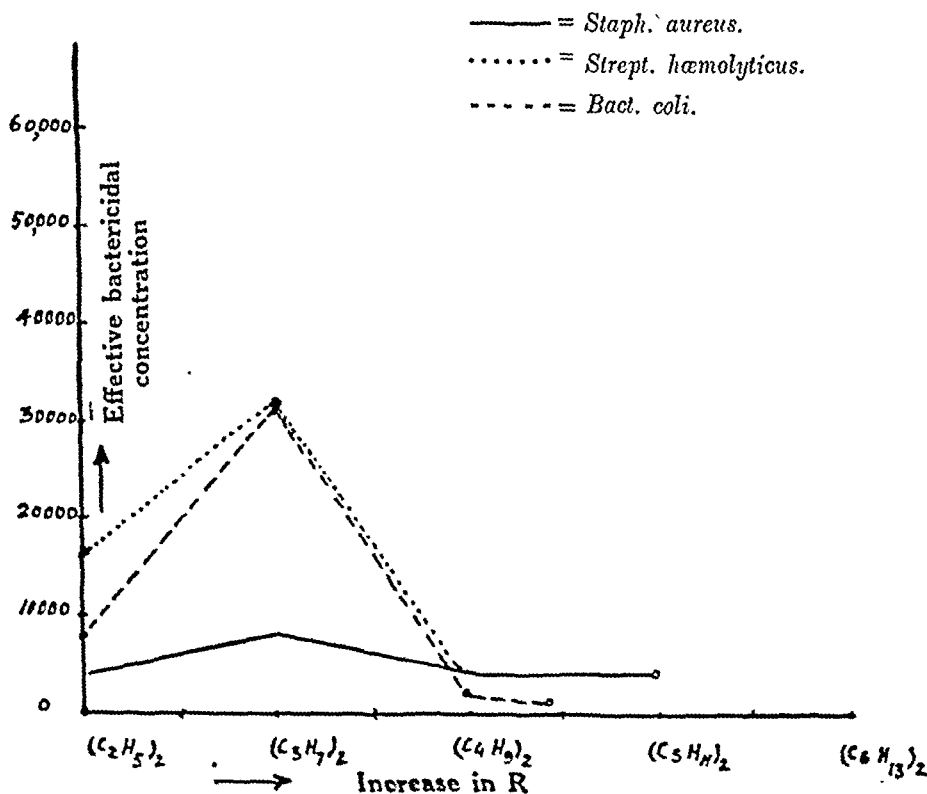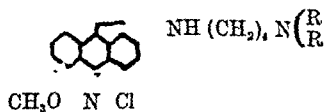
5. Quinacrine is inferior to almost all the compounds tested.

6. The activities of the compounds are enhanced in the presence of serum (*cf.* Table I with Table II). The compounds were found to be non-irritant.

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ANTISCORBUTIC DEFICIENCY DURING LACTATION AS A CAUSE OF INFANTILE SCURVY AND ' DENTAL DYSTROPHIES.

BY

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INTRODUCTION.

IT is now well established that the structure of the teeth and supporting structures may be markedly influenced by certain specific deficiencies in diet. Indeed structural changes may be produced almost at will by the withdrawal of one or other of the several essential food factors, particularly during the formative period of the teeth. Pre-natal nutrition is of the utmost importance if the teeth are to erupt without structural defects. Of perhaps still greater importance, at least in so far as the permanent dentition is concerned, is the diet of the mother during the lactation period.

The quality and quantity of the milk produced by the nursing mother is to a very high degree dependent on the character of her own diet. The work of Shukers et al. (1931) indicates a greatly increased caloric intake during lactation. Thus, the increased food requirement together with the tendency towards a negative calcium balance during this time would add to the danger of a general or specific food deficiency, with resulting harm to the offspring as well as to the mother during the nursing period. Of considerable importance in this connection is the fact that calcification of the deciduous and permanent teeth is primarily a function of post-natal life (Hess et al., 1932).

It is no longer thought that a child is necessarily well nourished simply because it is breast fed. Only to a very limited extent can the essential food factors be supplied from the mother's own body-stores. This is true particularly in the case of vitamins. Human milk is said to be low in vitamin D content and this would explain the fact that breast-fed children are by no means immune to rickets.

A sufficiency of the antirachitic vitamin D and of the antiscorbutic vitamin C is now recognized as essential for the proper utilization and fixation of calcium and phosphorus in the tissues.

It has been shown that the concentration of vitamin C in the milk of the nursing mother or lactating animal is largely dependent upon the diet received, and that the antiscorbutic vitamin in the food is rapidly secreted into the milk rather than stored in the body (Chick *et al.*, 1918 ; Dutcher *et al.*, 1920 ; Sherman, 1932 ; Hess, 1920 ; Hart *et al.*, 1920 ; Barnes and Hume, 1919). Therefore, the only dependable source of this important food factor must be the daily food supply. It is significant that vitamin C is one of the food elements in which the average diet of to-day is said by McCollum and Simmonds (1929) to show a tendency to be deficient. Aykroyd and Krishnan (1937) state that even in the villages during certain months of the year vitamin C is definitely deficient in Indian dietaries.

The literature contains many instances of scurvy outbreaks attributed to the practice of relying upon heated or pasteurized milk for the supply of antiscorbutic substance. It is noteworthy that frequently the only manifestations of the disease were fretfulness, anæmia, loss of appetite and retardation of growth.

It is, therefore, not improbable that cases of sub-acute or mild scurvy may be more common than is usually supposed, although not diagnosed as such on account of the absence of the typical symptoms of frank scurvy. When attention is focused on groups of children, as in the outbreaks reported, there is a greater likelihood of a true diagnosis being made.

In our experimental work with guinea-pigs (Day, 1933, 1934) we have found that the susceptibility to scurvy is subject to considerable individual variation. Similarly, Hess (1917*a*) reports that the antiscorbutic requirement for the maintenance of normal nutrition and growth is higher in some children than in others. Diagnosis of the disease may be rendered more difficult in view of the possibility of secondary infections being superimposed upon the primary nutritional disturbance. Conversely, as pointed out by Hess (1917*b*), latent scurvy may become suddenly manifest by the complication of intercurrent infection. Infantile scurvy is said to increase markedly the susceptibility to infection, nasal diphtheria, frequent attacks of grippe and other disorders being not uncommon. The slow onset of the disease clinically recognizable as scurvy constitutes an added danger to the young. The symptoms of frank scurvy may never develop, yet the child may have undergone a period of specific malnutrition with resulting dystrophies and with no clinical manifestations of deficiency other than a subnormal state of health. We have shown that in animals scurvy may exist in a latent or sub-acute form without the manifestation of clinical signs and symptoms. Even very small variations in the amounts of antiscorbutic received were followed by a very marked effect upon the tooth structure. The specific degeneration of the bone-marrow in infantile scurvy, thus preventing normal ossification, no doubt has its counterpart in the atrophy or degeneration of the pulp and dentine-forming cells of the tooth with resulting imperfect dentine formation. This is only to be expected at least during the formative period of the teeth, in view of the similarity of the disease in guinea-pigs and man.

Thus, partial antiscorbutic deficiency during lactation or in the diet of artificially fed infants may bring about an incipient scurvy, characterized by a subnormal state of health not clinically recognizable as scurvy but sufficient to cause dystrophies in the bones and teeth particularly during the formative period.

The specific needs of the pregnant animal for vitamin C and the effects upon the teeth of both the mother and the offspring of a partial deprivation of this vitamin during pregnancy have been demonstrated in our earlier investigations (Day, 1933).

The following investigation was designed to study the effects of a complete deprivation and of a partial deficiency of the antiscorbutic vitamin upon the body organism and dental tissues of the mother and offspring during the lactation period. Particular attention has been given to the rate of tooth growth, the mineral content of the bones and teeth and microscopic pathology of the dental tissues.

EXPERIMENTAL DATA.

From a very large group of virgin female guinea-pigs 30 were finally chosen for the initial portion of this investigation. The animals were divided into seven groups as follows :—

I. 6 lactating animals on basal diet alone.
II. 6 lactating animals on basal diet + 2 c.c. orange juice daily.
III. 6 lactating animals on basal diet + 8 c.c. orange juice daily.
IV. 3 lactating animals on basal diet + cabbage and lettuce.
V. 3 non-lactating animals on basal diet alone.
VI. 3 non-lactating animals on basal diet + 2 c.c. orange juice daily.
VII. 3 non-lactating animals on basal diet + 8 c.c. orange juice daily.

These animals with the addition of the 2 young from each mother in groups I to IV made a total of 72 animals in the seven main experimental groups. The experimental period for each of these animals was 20 days.

In addition, a subsidiary series of 16 mothers, each with two young, was used to obtain tissue sections for microscopy under varying degrees of antiscorbutic deficiency and over various periods of time ranging from 22 to 56 days. Including non-lactating controls the number of animals used for this portion of the investigation was, therefore, upwards of 120.

Although some difficulty was experienced in view of the difference in time necessary to effect successful impregnation an effort was made to keep the average weight variation of the animals, chosen for the experimental groups, as low as possible and within reasonable limits at the end of the gestation period. In order to eliminate another possible source of errors the number of young suckled by the mothers in each group was limited to two in each instance. Thus, the nutritive burden of each mother was as nearly as possible the same. The young also were chosen from the litters with a view to keeping their weight variation as low as possible.

The work of Donelson *et al.* (1931) indicates that there is a greater possibility of a negative calcium balance at the close of the second lactation than at the end

of the first. The danger of this variant entering into our results was circumvented by the use of virgin females throughout our groups so that all were having their first lactation.

The lactation period in guinea-pigs is approximately 25 to 30 days. Results of some trial experiments indicated that it would be impossible to sustain life under lactating conditions in a complete series on the chosen amounts of antiscorbutic for a period much longer than 20 days. This period of time was, therefore, used arbitrarily for the main series, in order that the mothers and young might be maintained on various vitamin C deficiencies for a uniform period for each group of the series.

The basal diet was designed to fulfil the two fundamental conditions, namely, that it must be quite free from antiscorbutic and must be in all other respects a complete dietary. The following basal ration was used :—

			Grammes.
Rolled oats (ground)	...	...	29·5
Skim milk powder	...	...	30·0
Wheat bran	...	...	...	29·5
Sodium chloride ...	...	...	1·0
Butter (freshly churned)	...	...	9·0

The milk powder was heated at 110°F. for three hours in shallow trays and stirred frequently. The ration was mixed in small quantities and stored in the ice-box.

In order to rule out the possibility of a deficiency of the A and D vitamins each mother (with the exception of those receiving cabbage) received 1 c.c. of cod-liver oil daily by mouth through a medicine dropper. In order to improve intestinal function thin strips of filter-paper were given with the ration and eaten readily. A vitamin E supplement was also given in the form of wheat-germ oil prepared by ether extraction and condensation.

Freshly extracted orange juice was administered daily directly into the mouth by pipette in graded doses as shown in Table I. For the purpose of obtaining growth curves all animals were weighed daily.

The upper and lower incisor teeth were marked by means of an ampoule file at the beginning of the 20-day experimental period and the growth measured twice during the lactating period. The average incisor growth per day for each group was thus obtained. The teeth of the three non-lactating controls for each group were likewise marked and measured and an average growth figure obtained.

At the end of the 20-day experimental period all the animals were chloroformed.

Radiographs were taken of the front chest wall, long bones and mandibles of representative animals of the various groups for purposes of comparison.

At necropsy sections were made of the mandibles, costochondral junction of the ribs and the knee-joints. One half of each mandible was dissected out, fixed in Müllers' solution, decalcified with nitric acid and embedded in paraffin.

Sections were then cut at right angles to the longitudinal axis of the mandible through, or just anterior to, the first molar tooth, and stained with hæmatoxylin and eosin. Sections from each of the 72 animals comprising the various groups were examined microscopically and photomicrographs made of representative sections from animals in all groups.

The femur and the incisor tooth (from that half of the mandible not used for sectioning) were then ashed and the percentage of mineral content calculated in each instance for both mother and young and similarly for the non-lactating animals.

Results and Discussion.

This experiment was designed with the object of investigating the effect of an absolute and relative antiscorbutic deficiency not only upon the lactating mothers but also on the young which were entirely dependent upon the mother's milk for their vitamin C supply.

As in the earlier work on pregnancy particular attention was given to the effect on the dental tissues ; but the opportunity was also taken to study the effect of this specific deficiency on the body-organism as a whole. Our results leave no room for doubt that the specific needs of the organism for antiscorbutic vitamin during the lactation period are equally as great as during the period of gestation.

Growth curves.—The weighing of the animals daily enabled us to obtain reliable growth curves.

Table I summarizes the average weights (in grammes) of the mothers in groups I to IV immediately after parturition, the final weights in grammes, the

Table I.

Mothers' weight.

Group.	Diet.	Number in group.	Days.	Average weight after parturition (grammes).	Final weight (grammes).	Average weight loss (grammes).	Percentage weight loss.
I.	Basal only ...	6	20	625	434	191	30·56
II.	Basal + 2 c.c. orange juice.	6	20	613	497	116	18·92
III.	Basal + 8 c.c. orange juice.	6	20	632	551	81	12·82
IV.	Basal + greens	3	20	689	656	33	5·03

average weight loss, and the percentage weight loss. It will be seen that the average and percentage weight loss during the experimental period of 20 days' lactation was inversely proportional to the amount of antiscorbutic administered.

Table II summarizes for groups I to IV the average weights of the young at birth, the final weights, the average weight gain and the percentage weight gain. It is noteworthy that in the case of the young the weight gains are directly proportional to the amount of antiscorbutic received by the mothers. The mother's milk was the sole source of vitamin C. It is assumed, therefore, that vitamin C was secreted into the milk of the mother in approximately the same ratio in which it was administered to her by mouth. Even when the mother received 8 c.c. of antiscorbutic in the form of orange juice, normal growth was not maintained by the young animals. This is evident from comparison of the growth figures for group III, and the control group IV which received greens in addition to the basal ration.

TABLE II.

Weight of young.

Group.	Diet.	Number in group.	Days.	Average weight at birth (grammes).	Average final weight (grammes).	Average weight gain (grammes).	Percentage weight gain.
I.	Milk of group I mothers.	8*	20	87	145	58	66·67
II.	Milk of group II mothers.	11*	20	84	147	63	75·00
III.	Milk of group III mothers.	11*	20	82	164	82	100·00
IV.	Milk of group IV mothers (greens).	6*	20	95	250	155	163·16

* Only those animals are included which were maintained on uniform diet for the full experimental period. The diet of some was varied or they were transferred to mothers of other groups in order to note the effect of such change or to obtain special tissue sections for microscopy.

The weight figures for these four groups are given in detail in Tables III, IV, V and VI. In order to obtain tissue sections for microscopy under varying conditions several animals were transferred to other groups for various periods or were given a different antiscorbutic dosage.

TABLE III.

Series 80. Guinea-pigs on basal diet only.

Animal number.	MOTHER'S.			FIRST YOUNG.			SECOND YOUNG.			MOTHER'S INCISOR GROWTH PER DAY (MM.).	
	Initial weight (grammes).	Final weight (grammes).	Weight loss (grammes).	Initial weight (grammes).	Final weight (grammes).	Weight gain (grammes).	Initial weight (grammes).	Final weight (grammes).	Weight gain (grammes).	Uppers.	Lowers.
31	575	419	156	63	110	47	63	124	61	0·262	0·275
21	545	342	203	66	96	30	66	133	67	0·212	0·250
10	635	447	188	100	182	82	99	162*	63	0·212	0·212
17	695	520	175	110	171	61	62	105*	43	0·263	0·250
16	608	505	103	113	210	97	91	191*	100	0·25	0·250
14	695	369	326	110	140	30	96	141*	45	0·187	0·200
Average ...	625	434	191	94	151	57	...	...	...	0·231	0·239
	Percentage weight loss 30·56			Percentage weight gain 60·64							

* Omitted from calculation, *cf.* Table II (footnote).

TABLE IV.

Series 80. Guinea-pigs on basal diet + 2 c.c. antiscorbutic.

Animal number.	MOTHER'S.			FIRST YOUNG.			SECOND YOUNG.			MOTHER'S INCISOR GROWTH PER DAY (MM.).	
	Initial weight (grammes).	Final weight (grammes).	Weight loss (grammes).	Initial weight (grammes).	Final weight (grammes).	Weight gain (grammes).	Initial weight (grammes).	Final weight (grammes).	Weight gain (grammes).	Uppers.	Lowers.
6	499	428	71	63	106	43	62	140	78	0·272	0·272
2	555	496	59	81	106	25	71	108	37	0·231	0·231
30	588	320	268	74	142	68	68*	140	72	0·235	0·250
26	660	582	78	88	138	50	71	157	86	0·224	0·250
23	716	660	56	121	200	79	101	188	77	0·250	0·276
11	660	499	161	103	187	84	94	161	67	0·200	0·237
Average ...	613	497	116	88	146	58	80	149	69	0·235	0·253
	Percentage weight loss 18·92			Percentage weight gain 65·91			Percentage weight gain 86·0				

* Omitted from calculation, *cf.* Table II (footnote).

TABLE V.

Series 80.   *Guinea-pigs on basal diet* + 8 *c.c. antiscorbutic.*

Animal number.	MOTHER'S.			FIRST YOUNG.			SECOND YOUNG.			MOTHER'S INCISOR GROWTH PER DAY (MM.).	
	Initial weight (grammes).	Final weight (grammes).	Weight loss (grammes).	Initial weight (grammes).	Final weight (grammes).	Weight gain (grammes).	Initial weight (grammes).	Final weight (grammes).	Weight gain (grammes).	Uppers.	Lowers.
5	750	708	42	91	211	120	89	214	125	0·145	0·237
28	575	402	173	68	96	28	66	91	25	0·302	0·276
18	656	525	131	88	157	69	86*	117	31	0·236	0·305
13	623	615	8	83	186	103	79	189	110	0·275	0·287
22	568	419	89	88	147	59	81	124	43	0·200	0·237
29	620	578	42	93	208	115	75	181	106	0·287	0·350
Average ...	632	551	81	85	167	82	78	160 (5)	...	0·241	0·282
	Percentage weight loss 12·82			Percentage weight gain 96·47			Percentage gain (5) 105·1				

* Omitted from calculation, *cf.* Table II (footnote).

TABLE VI.

Series 80.   *Guinea-pigs on basal diet* + *cabbage and lettuce* ad lib.

Animal number.	MOTHER'S.			FIRST YOUNG.			SECOND YOUNG.			MOTHER'S INCISOR GROWTH PER DAY (MM.).	
	Initial weight (grammes).	Final weight (grammes).	Weight loss (grammes).	Initial weight (grammes).	Final weight (grammes).	Weight gain (grammes).	Initial weight (grammes).	Final weight (grammes).	Weight gain (grammes).	Uppers.	Lowers.
27	595	558	37	97	247	150	93	212	119	0·262	0·262
15	725	695	30	93	265	172	86	241	155	0·239	0·259
9	747	715	32	101	280	179	100	257	157	0·276	0·329
Average ...	689	656	33	97	262	167	93	237	144	0·258	0·280
	Percentage weight loss 5·03			Percentage weight gain 172·16			Percentage weight gain 154·84				

Table II includes only those animals which were maintained on a uniform diet or with the same mothers throughout the whole experimental period.

The only variant in groups I to III in Table I was the amount of antiscorbutic administered and the only variant in groups I to III in Table II was the quality or vitamin C potency of the mothers' milk. The figures indicate the marked effect upon the growth of the nursing mothers and upon the young.
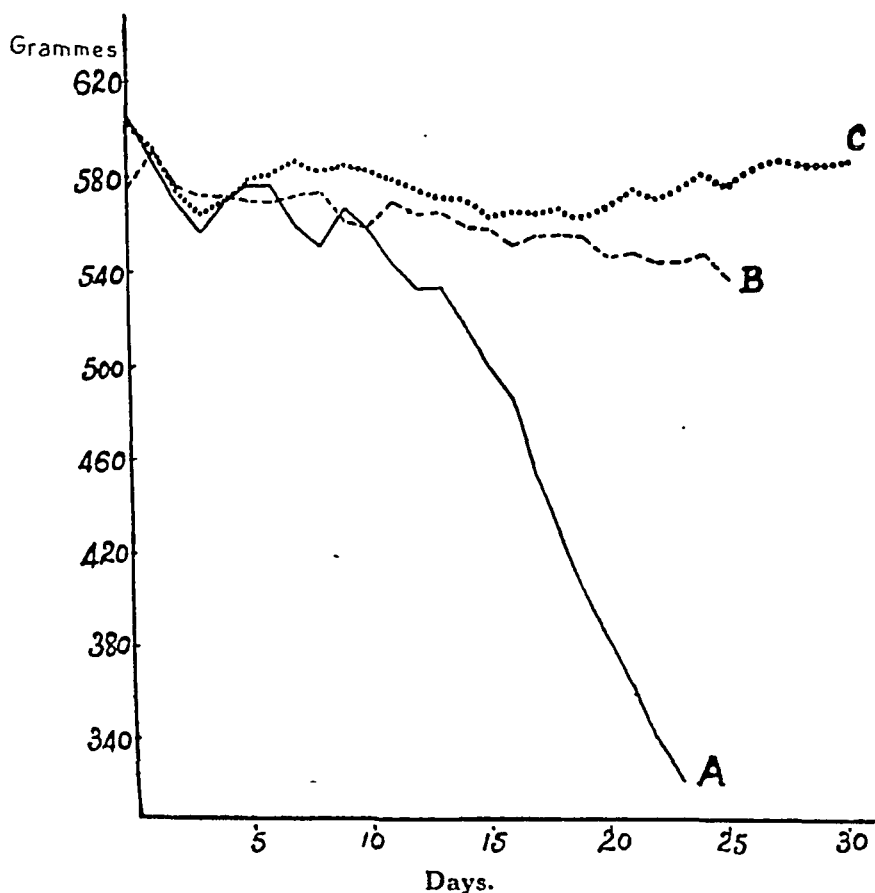
Fig. 1.

Fig. 1 represents the growth curves of lactating mothers in a supplementary series of animals. *A* is the average growth curve of three animals maintained on the basal diet alone. *B* is the average growth curve for four guinea-pigs maintained on 2 c.c. antiscorbutic. and *C* the average growth curve for six animals receiving 5 c.c. of antiscorbutic.

The effect upon the growth of the young is graphically illustrated in Fig. 2. Curves *A*, *B*, *C* and *D* respectively represent the average growth of (*A*) six young animals nursed by mothers receiving no antiscorbutic; (*B*) six young animals nursed by mothers receiving 2 c.c. antiscorbutic; (*C*) 10 young nursed by mothers receiving 5 c.c. antiscorbutic; and (*D*) 11 young nursed by mothers

Days.
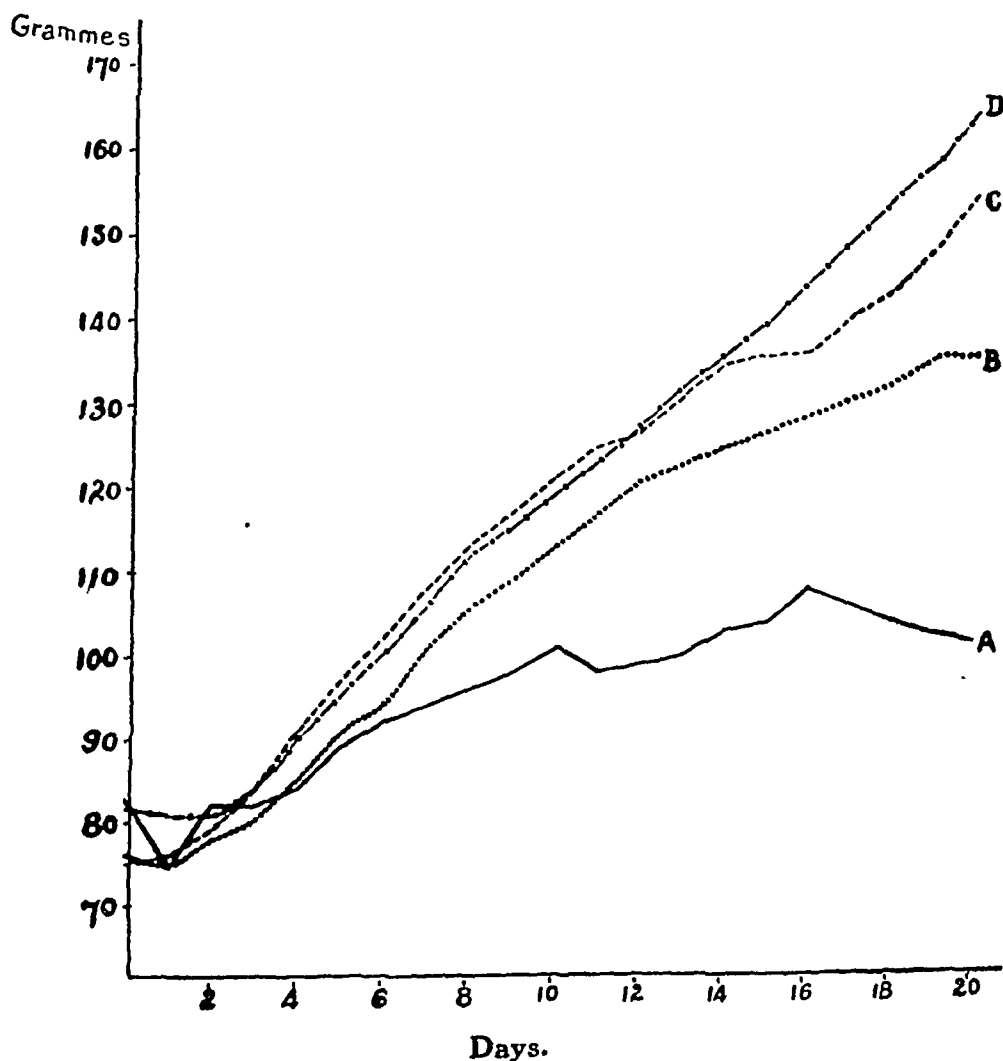
Fig. 2.

receiving 8 c.c. antiscorbutic for the full experimental period. These curves show a strikingly similar result to that obtained in the main groups.

In order to obtain growth curves of young under varying conditions mother No. 13 was maintained on the basal diet alone and received no antiscorbutic.

Young No. 1 was nursed by the mother until she died on the 24th day. The young then subsisted on the vitamin C-free basal diet for a further 16 days when it also died. The growth curve is represented by curve *X* in Fig. 3 :—
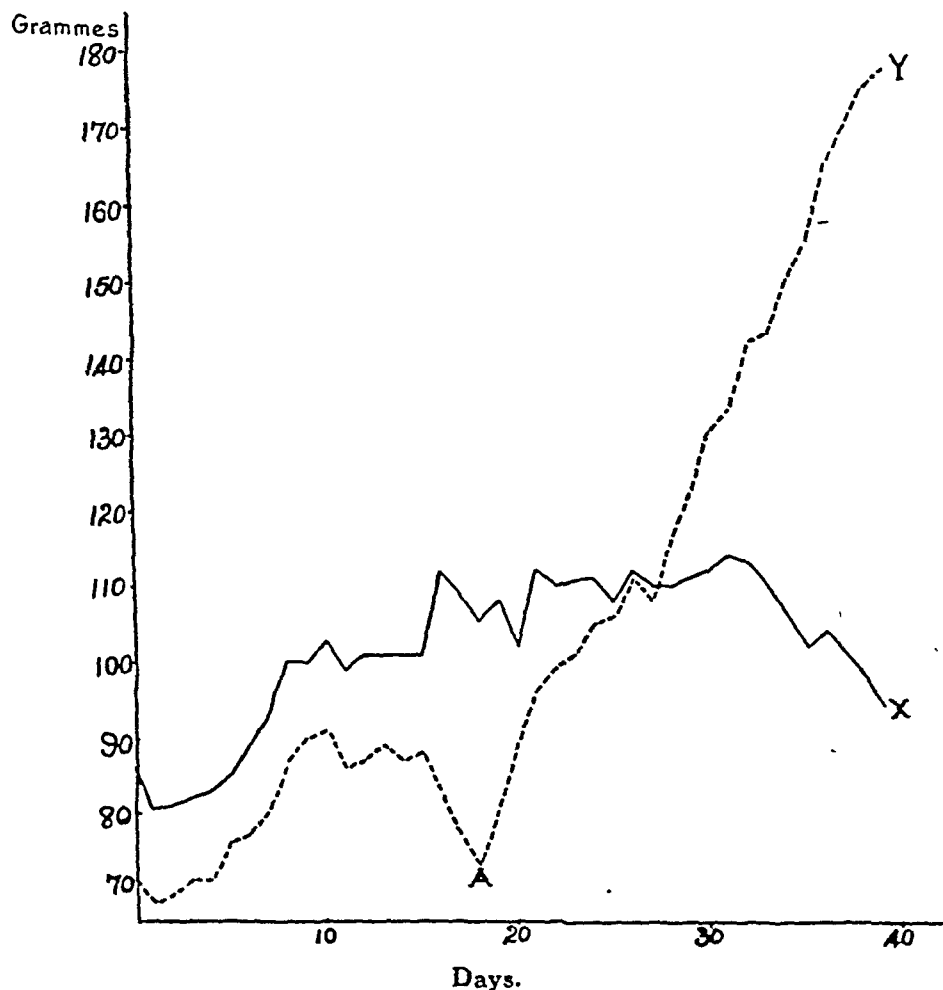
Fig. 3.

Young No. 2 was maintained on the milk of mother No. 13 for 18 days and from then onwards received by mouth a supplement of 2 c.c. of orange juice daily (curve *Y*, Fig. 3). Point *A* in curve *Y* represents the point at which the antiscorbutic supplement was administered. On the 39th day when young No. 1 died at 94 g. weight, young No. 2, although it had been steadily losing weight from

the 15th day, increased in weight so rapidly from the first dose of antiscorbutic that it weighed 177 g. when chloroformed on the 39th day.

For purposes of comparison of the microscopic tooth picture only three animals were used in the non-lactating control groups. This number was too small to give reliable growth curves in so short a period of 20 days. However, these animals showed an average weight gain in all groups in contrast to the weight loss in the lactating groups.

TOOTH GROWTH.

The teeth of guinea-pigs are of persistent growth. Advantage was taken of the fact to measure the growth of the incisors of the mothers in the different groups in order to check the relative effect in this respect of various degrees of deprivation of vitamin C.

The incisor growth of non-lactating controls on similar dosages of antiscorbutic was also measured.

The method employed was as follows :—

At the beginning of the experimental period both upper and lower incisor teeth of each animal were carefully scored with a fine ampoule file at 2 mm. from the gingival margin. It was necessary to measure growth from the cervical margin in order to eliminate the error which would otherwise arise due to the constant attrition and wearing of the incisal edge.

Measurements were made with very fine dividers after 10 days and again when the animals were chloroformed on the final day of the experimental period. The measurements for the individual animals are given in Tables III, IV, V and VI.

For lactating animals these figures are summarized in Table VII. Although, as might be expected, there was considerable individual variation, it was found that the average growth per day in both the upper and lower jaw was directly proportional to the amount of antiscorbutic administered. The average growth of the lower incisors was in all cases greater than that of the uppers. The mean average incisor growth per day was, therefore, also proportional to the amount of vitamin C in the diet.

TABLE VII.

Growth of incisor (mothers).

Group.	Diet.	Number in group.	AVERAGE INCISOR GROWTH IN MM.		Mean average incisor growth per day (upper and lower).
			Uppers.	Lowers.	
I.	Basal only	6	0·231	0·239	0·235
II.	Basal + 2 c.c. orange juice	6	0·235	0·253	0·244
III.	Basal + 8 c.c. orange juice	6	0·241	0·282	0·262
IV.	Basal + greens	3	0·258	0·280	0·269

These remarkably constant results would thus seem to indicate that the rate growth of the incisor teeth of guinea-pigs is a precise indication of the degree of scurvy.

Three non-lactating controls for each of groups I, II and III showed the following average growth rate for the incisors expressed in millimetres per day :—

Group I (no antiscorbutic)	Uppers Lowers	0·236 0·298	Mean average	0·267
Group II (2 c.c. antiscorbutic)	Uppers Lowers	0·249 0·305	Mean average	0·277
Group III (8 c.c. antiscorbutic)	Uppers Lowers	0·264 0·312	Mean average	0·288

These animals were approximately the same age and weight as those in the lactating groups. It will be seen that the incisor growth rate is again proportional to the amount of antiscorbutic received, and, moreover, would appear to be significantly greater in the non-lactating than in the lactating groups. From the point of view of incisor growth it should, therefore, be reasonable to assume a specific response of the teeth to the presence or absence of the antiscorbutic vitamin.

The proportionately reduced growth rate in the lactating as compared with the non-lactating groups would appear to indicate an intensified effect of a lack of this vitamin during lactation.

MINERAL CONTENT OF BONES AND TEETH.

It was thought that very little significance would attach to the figures in relation to the mineral content of the bones and teeth in the various groups as the experimental period was comparatively short.

It was, however, decided to ash one femur and one incisor of each of the mothers and the young animals.

Method.—The bones were ashed individually and the incisors of each group ashed together and an average taken.

The femur or incisors to be ashed were placed in porcelain crucibles which had previously been heated to a constant weight and weighed.

These were then dried in an incubator at a temperature of 100°C. for 8 hours, cooled in a desiccator for 5 minutes and weighed, and the weight of dried bone or incisor calculated. The organic matter was then burnt off by heating over a burner for 30 minutes at a dull red heat until the ash was grey or white.

This was then removed from the muffle, cooled in the air for 3 minutes, then transferred to the desiccator, cooled and weighed.

The results obtained in the various groups (*a*) basal ration only ; (*b*) 2 c.c. antiscorbutic; (*c*) 8 c.c. antiscorbutic; and (*d*) greens *ad lib.* are set out in detail in Tables IX and X.

The average per cent ash content of the incisors of the three non-lactating animals in each group receiving respectively 0 c.c., 2 c.c. and 8 c.c. of antiscorbutic was found to be 78·03, 77·97 and 77·17.

Table XII summarizes the results given in Tables VIII, IX, X and XI :—

TABLE VIII.

Per cent ash.

NO ANTISCOR-BUTIC.	MOTHER.		FIRST YOUNG.		SECOND YOUNG.	
Animal number.	Femur.	Incisor.	Femur.	Incisor.	Femur.	Incisor.
31	60·64	...	49·15	...	47·53	...
21	61·55	...	47·29	...	48·59	...
10	61·66	...	47·37	...	45·55*	...
17	62·17	...	46·29	...	42·79*	...
16	60·02	...	45·26	...	44·00*	...
14	62·18	...	41·53	...	42·06*	...
Average ...	61·37	78·16	46·15	74·93	48·06	...

* Omitted from calculation, *cf.* Table II (footnote).

TABLE IX.

Per cent ash.

2 C.C. ORANGE JUICE.	MOTHER.		FIRST YOUNG.		SECOND YOUNG.	
Animal number.	Femur.	Incisor.	Femur.	Incisor.	Femur.	Incisor.
6	60·92	...	41·17	...	48·25	...
2	61·94	...	43·93	...	40·45	...
30	62·06	...	48·12	...	48·67*	...
26	57·40	...	47·85	...	49·29	...
23	59·56	...	44·23	...	49·01	...
11	56·39	...	46·13	...	42·78	...
Average ...	59·71	77·45	45·24	73·71	45·96	75·14

* Omitted from calculation, *cf.* Table II (footnote).

TABLE X.

Per cent ash.

8 C.C. ORANGE JUICE.	MOTHER.		FIRST YOUNG.		SECOND YOUNG.	
Animal number.	Femur.	Incisor.	Femur.	Incisor.	Femur.	Incisor.
5	60·25	...	46·60	...	47·09	...
28	60·19	...	34·85*	...	35·37	...
18	62·31	...	43·20	...	46·20	...
13	62·33	...	49·32	...	49·80	...
22	59·88	...	41·81	...	41·34	...
29	58·72	...	58·77	...	51·27	...
Average ...	60·61	77·37	46·87	75·19	47·37	...

* Omitted from calculation, *cf*. Table II (footnote).

TABLE XI.

Per cent ash.

CABBAGE AND LETTUCE *ad lib.*	MOTHER.		FIRST YOUNG.		SECOND YOUNG.	
Animal number.	Femur.	Incisor.	Femur.	Incisor.	Femur.	Incisor.
27	62·43	...	52·19	...	51·44	...
15	62·12	...	52·29	...	51·55	...
9	60·55	...	50·27	...	52·04	...
Average ...	61·70	77·19	51·58	73·99	51·68	74·60

TABLE XII.

Per cent ash.

Group.	Diet.	MOTHER.			YOUNG.		
		Number of animals.	Femur.	Incisor.	Number of animals.	Femur.	Incisor.
I.	Basal only ...	6	61·37	78·16	8	46·63	74·93
II.	Basal + 2 c.c. orange juice.	6	59·71	77·41	11	45·57	74·36
III.	Basal + 8 c.c. orange juice.	6	60·61	77·37	11	44·86	75·07
IV.	Basal + greens ...	3	61·70	77·19	6	51·63	74·30

On the basis of these figures it would seem that the percentages of total minerals in the bones or teeth is in no way proportional to the amount of antiscorbutic administered. and that the effect upon the mineralization of the bones and teeth must be qualitative rather than quantitative. It must be remembered, however, that the experimental period, namely 20 days, decided upon for reasons above stated, was comparatively short and that only tissue laid down subsequent to the commencement of the experimental period would be affected by the dietary deficiencies. Furthermore, on a diet which is completely scorbutic the tooth growth soon decreases, and finally ceases altogether; and owing to pain and tenderness in the tempero-mandibular joints and the looseness of the teeth through alveolar bone changes the attrition is not nearly as pronounced. This would explain the observed phenomenon of a greater degree of macroscopical softening of the molars and incisors in animals on only partially deficient diets for a greater length of time when perhaps the whole dentine had been replaced by a deficient product of the odontoblasts and attrition was still active.

Fig. 4.

Fig. 4 shows the macroscopic appearance of this dentine-softening occurring in a partially deficient animal. The dentine towards the palatal aspect of the upper incisor teeth has become of a soft and cheesy consistency and 'feathered' under the stress of mastication against the enamel-covered edge of the lower incisors.

Although it is possible, for reasons already stated, that the total mineral content may not be appreciably altered within the limited experimental period

of 20 days, yet microscopical examination reveals marked dentine changes and faulty calcification probably due to a lack of fusion of the calcospherites.

MICROSCOPIC PATHOLOGY.

Transverse sections of the decalcified incisor roots stained with hæmatoxylin-eosin were prepared of all lactating and non-lactating animals and their young and photomicrographs taken of a number of the representative sections of the various groups.

A study of the photomicrographs clearly demonstrates the profound effect of hypovitaminosis-C on the dental tissues, and illustrates the importance of an adequate maternal diet during the period of lactation, not only for the protection of the mother but also to ensure adequate amounts of vitamin C in the milk for effective protection of the young.

Although the mother's diet may be only slightly deficient in vitamin content the antiscorbutic potency of the milk is proportionally reduced as evidenced by the marked scorbutic changes in the dental tissues of the young. Moreover, these pathological tissue changes run parallel to the other observed and previously noted phenomena of . interference with body-growth and tooth-growth, even in the absence of scurvy symptoms, clinically recognizable as such.

The pathological tissue changes brought about on various deficiencies of anti-scorbutic in lactating mothers and their young are illustrated in the photomicro-graphs and radiographs on Plates I to VIII.

Figs. 5 to 11 (Plates I to III) are photomicrographs of tissue sections taken from guinea-pigs maintained on the basal diet *plus* 5 c.c. antiscorbutic in the form of orange juice, and from their young under varying conditions. On the basis of 3 c.c. for 300 g. body-weight (Sherman's estimate) the amount of vitamin C was only slightly deficient. On such slight deficiencies the mother may show no clinical evidence of scurvy and at autopsy may appear well nourished with no signs of scurvy and no observable hæmorrhages or increased brittleness of the hard structures ; yet microscopical examination of the teeth of both mother and young shows a condition typical of latent scurvy. Disorganization of pulp-tissue and disturbance and degeneration of both odontoblasts and ameloblasts with consequent defective dentine and enamel structure are apparent as evidence of a condition of sub-scurvy.

Figs. 12 to 16 (Plates III and IV) are sections from lactating animals (and their young) maintained on 2 c.c. antiscorbutic in addition to the basal ration. This represents approximately 40 per cent of the protective dose and the photomicrographs show that degenerative changes are proportionately more pronounced.

These photomicrographs prove conclusively that the protection of the young is dependent upon an adequate supply of protective substance in the diet of the lactating mother, that the vitamin C potency of the milk is directly proportional to the amount of antiscorbutic in the mother's diet, and that the effects of a deficiency upon the teeth of the young, though perhaps varying in degree, parallel very closely those observed in the dental tissues of the mother.

On the basis of these figures it would seem that the percentages of total minerals in the bones or teeth is in no way proportional to the amount of anti-scorbutic administered, and that the effect upon the mineralization of the bones and teeth must be qualitative rather than quantitative. It must be remembered, however, that the experimental period, namely 20 days, decided upon for reasons above stated, was comparatively short and that only tissue laid down subsequent to the commencement of the experimental period would be affected by the dietary deficiencies. Furthermore, on a diet which is completely scorbutic the tooth growth soon decreases, and finally ceases altogether; and owing to pain and tenderness in the tempero-mandibular joints and the looseness of the teeth through alveolar bone changes the attrition is not nearly as pronounced. This would explain the observed phenomenon of a greater degree of macroscopical softening of the molars and incisors in animals on only partially deficient diets for a greater length of time when perhaps the whole dentine had been replaced by a deficient product of the odontoblasts and attrition was still active.

FIG. 4.

Fig. 4 shows the macroscopic appearance of this dentine-softening occurring in a partially deficient animal. The dentine towards the palatal aspect of the upper incisor teeth has become of a soft and cheesy consistency and 'feathered' under the stress of mastication against the enamel-covered edge of the lower incisors.

Although it is possible, for reasons already stated, that the total mineral content may not be appreciably altered within the limited experimental period

PLATE I.

FIG. 5.
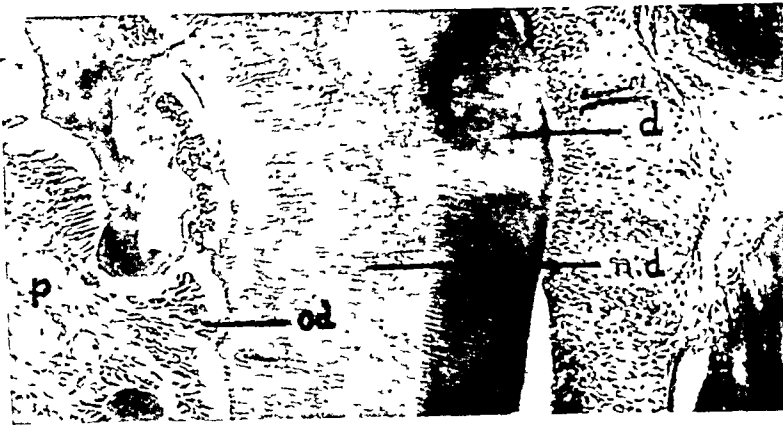
FIG. 6.

FIG. 7.

Fig. 5. A transverse section through the roots of an incisor of a fully protected animal. This high-power photomicrograph shows a normal line of odontoblasts and of ameloblasts, normal bands of dentine and enamel and no pulpal abnormality. The section is from one of the young (chloroformed at 40 days of age) of a mother maintained on the basal ration *plus* cabbage and lettuce *ad lib*. At autopsy there were no hæmorrhages into the ribs, joints or intestines and no brittleness of bones or teeth. The initial weight was 62 g. and the final weight 235 g. (× 100.)

„ 6. An incisor cross-section from a mother maintained on the basal ration *plus* 5 c.c. antiscorbutic (orange juice) per diem. The weight at the beginning of the period of lactation was 535 g. and when chloroformed after 30 days the weight was 586 g. On the accepted basis of 3 c.c. for 300 g. body-weight the amount of vitamin C was, therefore, only slightly deficient. At autopsy the mother appeared well nourished with no symptoms of scurvy and with no observable hæmorrhages or increased brittleness of the hard structures; yet the photomicrograph of the tooth section shows a condition typical of latent scurvy. There is considerable disorganization of the pulp tissue, irregularity and shortening of the odontoblasts and since the beginning of the experimental dieting period a defective and more lightly stained band of dentine has been laid down with bone-like projections into the pulp. (× 100.)

„ 7. An incisor cross-section through the region of the symphysis of the young of a mother maintained on 5 c.c. of orange juice in addition to the basal ration. This young animal survived for only 35 days and the photomicrograph shows typical scorbutic degenerative changes in the pulp. The odontoblasts have almost entirely disappeared and within the outer band of normal dentine has been laid down a band of irregular ' osteodentine ' with finger-like projections into the pulp. (× 28.)

Abbreviations :—e, enamel; am, ameloblasts; d, dentine; od, odontoblasts; nd, new dentine; p, pulp; bv, blood vessel; v, vacuolation; oz, odontogenic zone; J, jaw bone; tc, tomes' canals; pb, pulp bone; cd, calcific deposit.

Fig. 8. An incisor cross-section of a young guinea-pig nursed by a mother maintained during lactation on 5 c.c. of orange juice in addition to the basal ration. The young was chloroformed with the mother on the 32nd day after birth. Comparatively normal bands of dentine and enamel are shown but the odontoblasts are becoming shortened and irregular. ($\times$ 210.)

„ 9. A photomicrograph of an incisor cross-section from the second young of the mother described in connection with Fig. 8. This young animal was maintained on the mother's milk during the lactation period and thereafter on the basal ration alone until death after 56 days from birth. A scurvy condition with degenerative changes similar to those described in connection with Fig. 7 is shown. ($\times$ 28.)

„ 10. An incisor cross-section of the second young of another mother of the 5 c.c. group. The young animal was largely protected during the lactation period by the vitamin C secreted in the mother's milk. The mother was chloroformed with the two young after 40 days: but from the 22nd day the second young received a small supplement of orange juice (1 c.c. per diem) and showed no clinical or autopsy evidence of scurvy. The tooth picture, however, shows a condition of sub-scurvy as evidenced by the irregular and defective band of dentine. Furthermore, the ameloblast line shows considerable disturbance with irregular masses of abnormal enamel laid down and some ameloblasts enclosed in the irregular deposit. This shows a calcification anomaly somewhat similar in nature to the irregular and amorphous masses of dentine laid down in the pulp in sub-scurvy of the more severe type. ($\times$ 210.)

Abbreviations :—**e,** enamel ; **am,** ameloblasts ; **d,** dentine ; **od,** odontoblasts ; **nd,** new dentine ; **p,** pulp ; **bv,** blood vessel ; **v,** vacuolation ; **oz,** odontogenic zone ; **j,** jaw bone ; **tc,** tomes' canals ; **pb,** pulp bone ; **cd,** calcific deposit.

Fig. 11. An incisor cross-section of the second young of another mother of the 5 c.c. group. The mother was chloroformed 30 days after parturition. For the first 30 days the young was obtaining vitamin C from the mother's milk. From that time onwards until death on the 52nd day it subsisted on the basal ration alone.

These diet changes are graphically represented in the photomicrograph. The outer band of normal dentine was laid down *in utero*, i.e. during the pre-lactation period, the second defective band during the period of lactation when the animal was partly protected and the inner calcific mass which completely fills the pulp cavity, after the 30th day when the animal was completely deprived of vitamin C by subsisting on the basal ration alone. The ameloblast line is also disturbed and an irregular calcific mass is laid down in place of the normal enamel. On the 52nd day the animal showed clinical signs of scurvy with hæmorrhagic gums and loosening of the molar teeth, and at autopsy marked hæmorrhages into the ribs and joints and brittleness of the bones. ($\times$ 28.)

„ 12. A photomicrograph of the mandible and incisor cross-section from a lactating guinea-pig maintained on the basal diet *plus* 2 c.c. of orange juice per diem. This represents approximately 40 per cent of the protective dose of antiscorbutic vitamin (Sherman's estimate), the mother's weight being 522 g. at the beginning of lactation. As in the 5 c.c. group each animal in the 2 c.c. group nursed two young. This mother was chloroformed after 32 days. The photomicrograph shows a condition typical of a partially protected animal. Note the thin outer layer of normal dentine laid down before the beginning of the experimental dieting on parturition and within that a layer of defective dentine deposited by degenerating odontoblasts. The odontoblast line is becoming irregular and there is a wide predentine and some calcific deposit already appearing within the pulp. At necropsy the mother appeared under nourished but there was very little hæmorrhage into the tissues or brittleness of the bones yet the degenerative tooth changes were well marked. ($\times$ 28.)

„ 13. An incisor cross-section of the second young of the mother described in Fig. 12 chloroformed with the mother. This shows a tooth picture almost identical with that of the mother. Note the degeneration and irregularity of the odontoblasts, the outer normal band and inner defective band of dentine. In all these cases the loose connection between the normal band and the inner defective band deposited during the experimental dietary period is ~rent. The two bands usually become separated during th~ ($\times$ 210.)

*Abbreviations :—*e, enamel ; **am**, ameloblasts ; **d**, der� ·lasts〗 c ;
p, pulp ; **bv**, blood vessel ; **v**, vacu〗 ·enic〗 ;
tc, tomes' canals ; **pb**, pulp bone 〗

PLATE III.

Fig. 11.
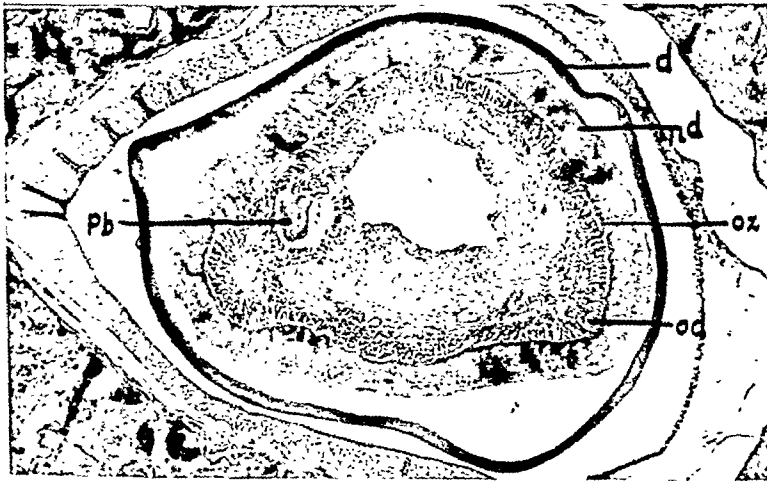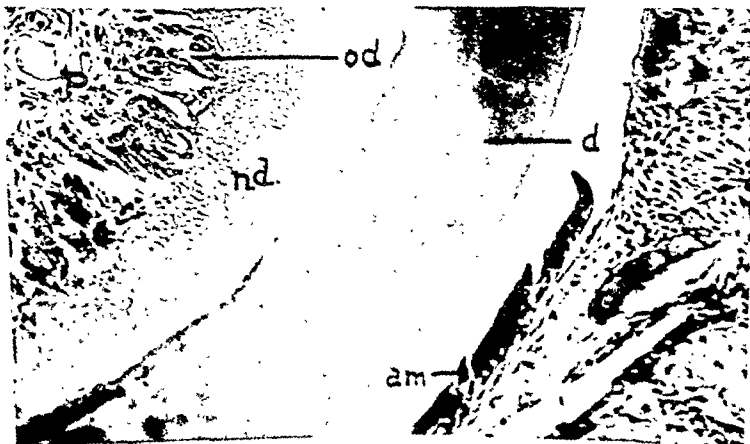
Fig. 12.

Fig. 13.

PLATE IV.

Fig. 14.
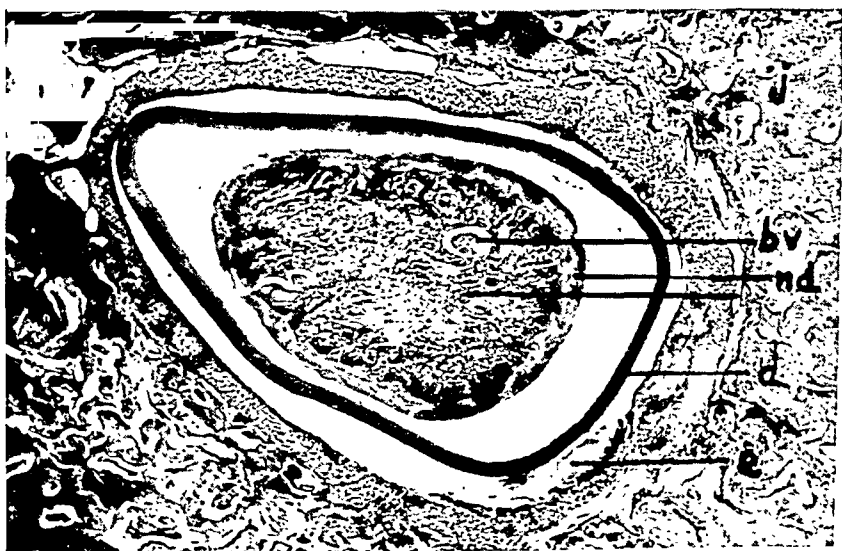
Fig. 15.
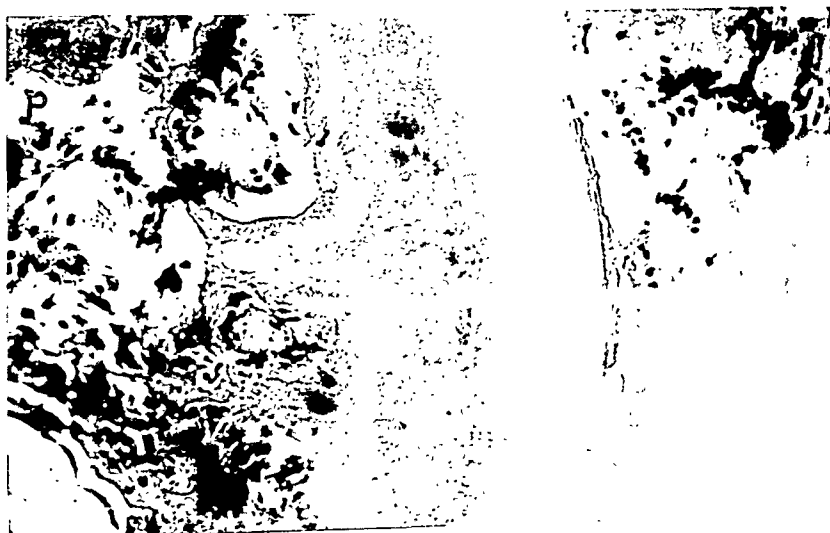
Fig 16.

Fig. 14. An incisor cross-section of the first young of the mother described under Fig. 12. This young animal was nursed by the mother until the latter was killed on the 32nd day following parturition, and from then until it died on the 46th day subsisted on the basal ration alone. This shows a somewhat similar tooth picture to that of its litter-mate (Fig. 13) but the degenerative changes are more pronounced. The odontoblasts have become shorter and more irregular and are becoming grouped around and in some cases enclosed within calcific deposits in the pulp. At autopsy the animal showed marked evidence of scurvy, hæmorrhage into the ribs, joints and intestines and brittleness of the bones. (× 210.)

Figs. 15 and 16. Incisor cross-sections of the two young of another lactating guinea-pig of the 2-c.c. group. Fig. 15 shows a tooth picture identical with Fig. 13 and is taken from a young animal chloroformed with the mother after 33 days of experimental dieting. Fig. 16 is from the second young which died after 44 days. During the last 11 days, i.e. since being deprived of its mother's milk on the 33rd day, it had subsisted on the basal ration only. The experimental conditions, survival times and tooth pictures of these two animals are almost identical with those of the young animals described in Figs. 13 and 14 respectively. (Fig. 15 = × 28; Fig. 16 = × 210.)

Abbreviations :—**e,** enamel ; **am,** ameloblasts ; **d,** dentine ; **od,** odontoblasts ; **nd,** new dentine ; **p,** pulp ; **bv,** blood vessel ; **v,** vacuolation ; **oz,** odontogenic zone ; **j,** jaw bone ; **tc,** tomes' canals ; **pb,** pulp bone; **cd,** calcific deposit.

Fig. 17. Photomicrograph of an incisor cross-section of a lactating mother maintained since parturition on the basal ration alone, i.e. with no vitamin C supplement, until death after 23 days. A typical scurvy condition is seen with degeneration of the dentine-forming cells and pulp-tissue. (× 28.)

,, 18. A similar cross-section from the first young of the mother (Fig. 17). This young was chloroformed on the mother's death. The photomicrograph shows a very thin dentine band and some degenerative changes in the odontoblast line; but in the short period of 23 days these changes are not nearly so marked as in the mother. (× 100.)

,, 19. An incisor cross-section of the second young of mother (Fig. 17). Following the death of the mother this animal subsisted on the basal ration alone until it died on the 36th day. Here the degenerative changes are well marked. The odontoblast line has disappeared, the dentine band is very thin, and a small amount of calcific tissue has been laid down on the margin of the pulp and hæmorrhages have occurred into it. The normal ameloblast line has also become disorganized and irregular calcific masses have been deposited in place of the normal enamel. On the death of the mother this animal received a ration of cod-liver oil to ensure a sufficiency of vitamins A and D. (× 28.)

Abbreviations :—**e,** enamel; **am,** ameloblasts; **d,** dentine; **od,** odontoblasts; **nd,** new dentine; **p,** pulp; **bv,** blood vessel; **v,** vacuolation; *oz,* odontogenic zone; *j,* jaw bone; **tc,** tomes' canals; **pb,** pulp bone; **cd,** calcific deposit.
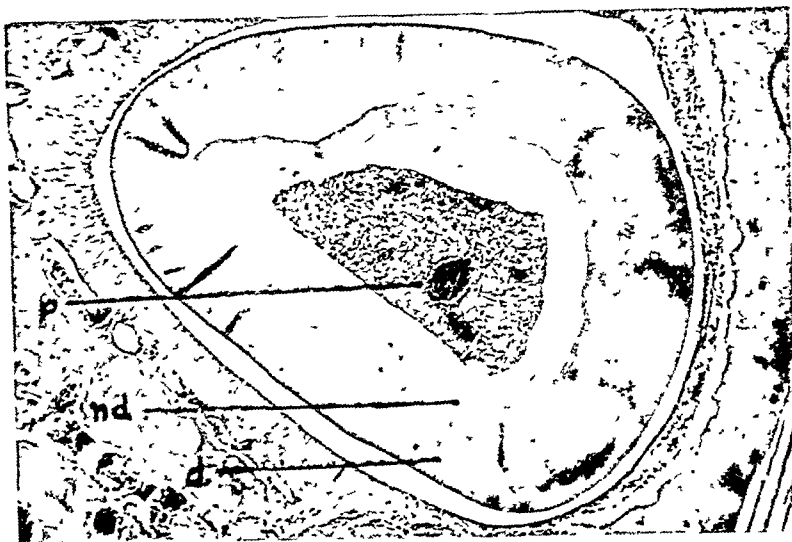
PLATE V.

Fig. 17.
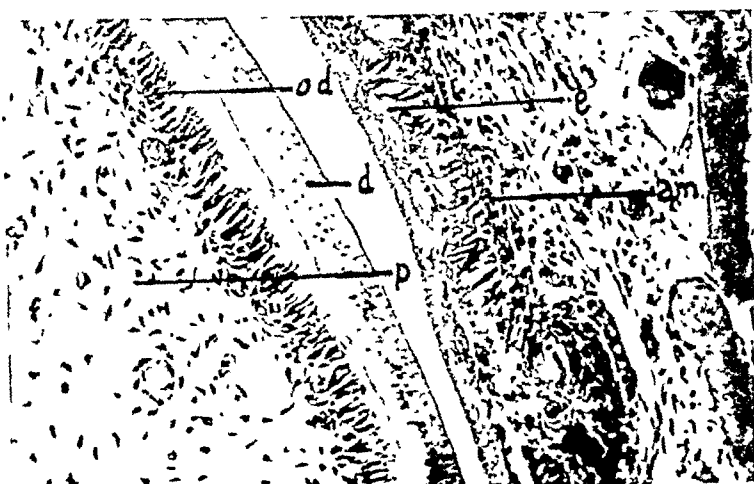
Fig. 18.
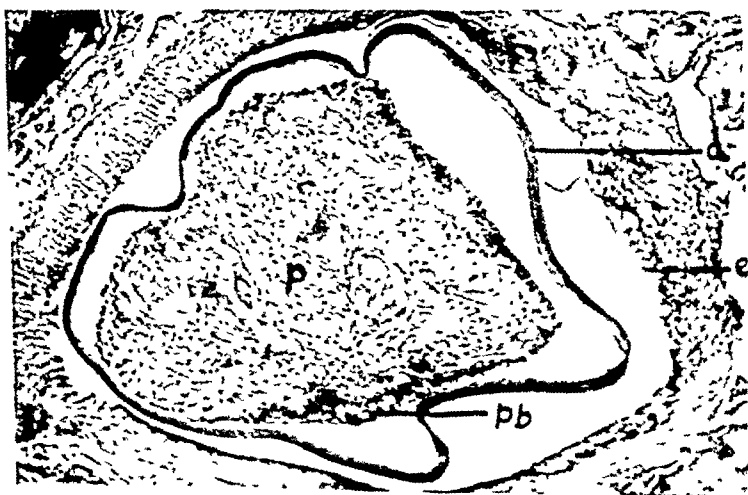
PLATE VI.
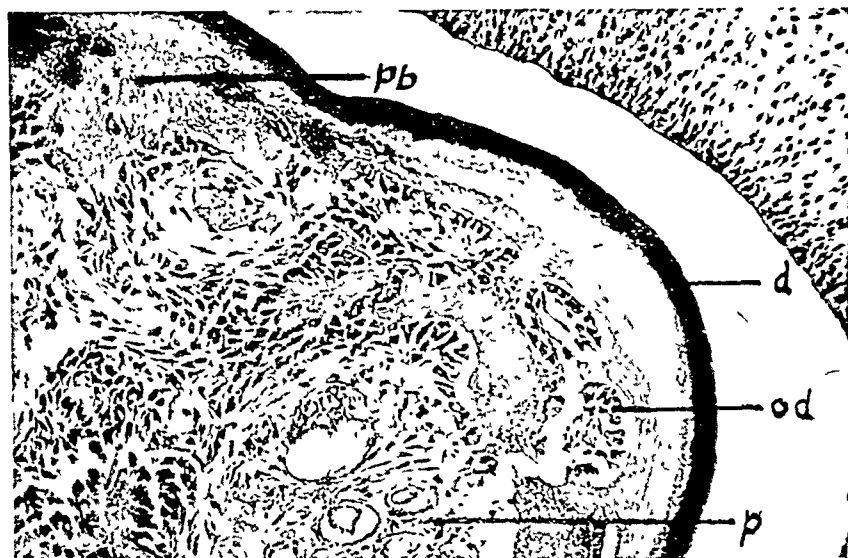
Fig. 20.
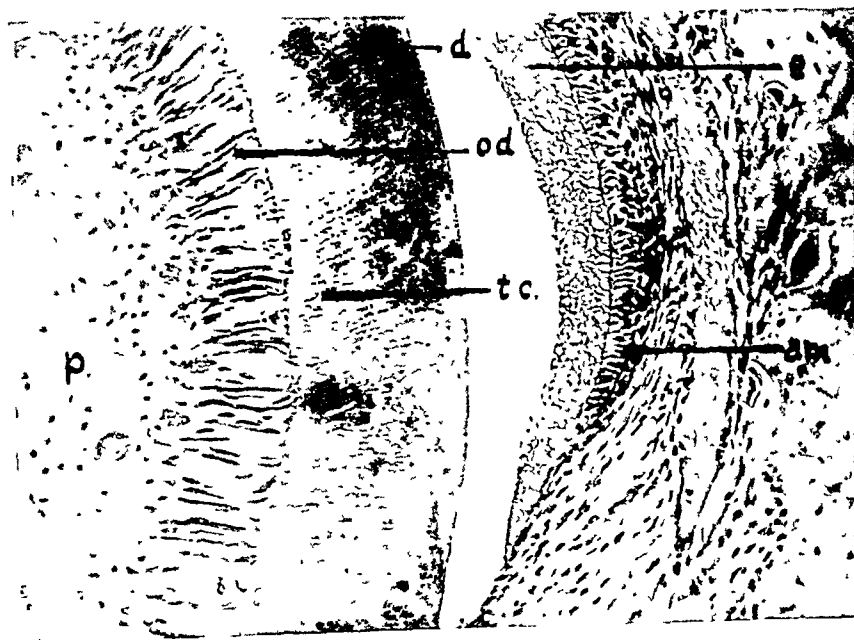
Fig. 21.

Fig. 20. A section through the mandible of the first young of another mother maintained during lactation on the basal ration only. The mother died after 24 days, and the young survived for 15 days longer during which time it subsisted on the basal ration alone with a supplement of cod-liver oil. The section shows that normal dentine is no longer being laid down and typical scorbutic pulpal changes are apparent. At autopsy this animal had a high Sherman score with severe hæmorrhages into the ribs and joints and brittleness of the bones and teeth. The molar teeth were very loose indicating degenerative changes in the supporting structures. (× 100.)

„ 21. A section from the second young whose litter-mate is shown in Fig. 20. This animal lived under identical conditions with the first young until the 18th day, but thereafter until chloroformed on the 39th day it received a supplement of 2 c.c. orange juice *per diem.* The whole tooth picture from pulp to enamel presents a perfectly normal appearance. At autopsy this animal showed a negative Sherman score with no hæmorrhages and no looseness of the molar teeth. (× 210.)

The growth curves of these two animals (Figs. 20 and 21) have been graphically illustrated and discussed (*see* Fig. 3).

Abbreviations :—**e,** enamel ; **am,** ameloblasts ; **d,** dentine ; **od,** odontoblasts ; **nd,** new dentine ; **p,** pulp ; **bv,** blood vessel ; **v,** vacuolation ; **oz,** odontogenic zone ; **j,** jaw bone : **tc,** tomes' canals ; **pb,** pulp bone ; **cd,** calcific deposit.

Fig. 22. A radiograph of a leg fracture which occurred spontaneously in the cage and noted as a common occurrence with scorbutic guinea-pigs.

,, 23. A radiograph of the same fracture (Fig. 22) united by a firm callus without splinting following the administration of antiscorbutic.
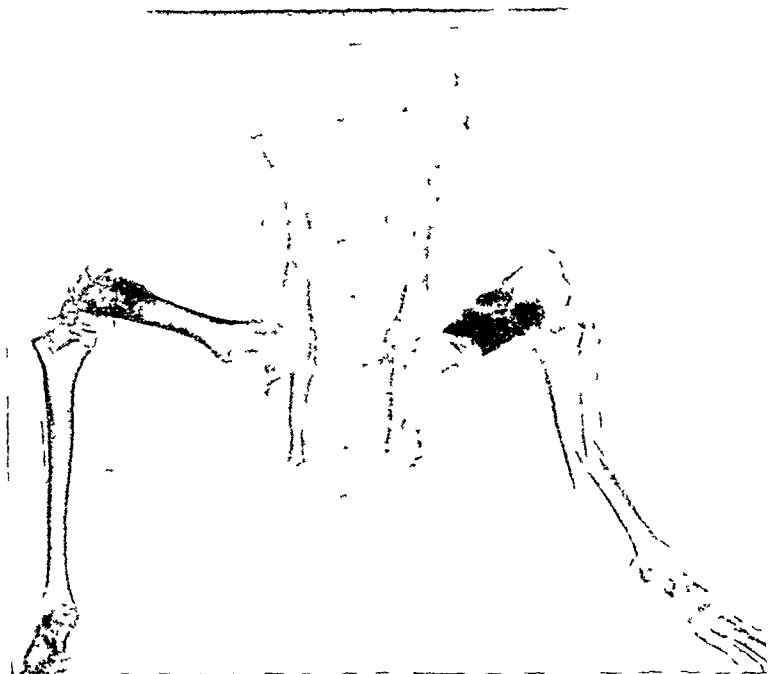
PLATE VII.

Fig 22.

Fig. 23.

Basal ration + greens
  ad lib.

Basal ration + 5 c c.
  antiscorbutic.
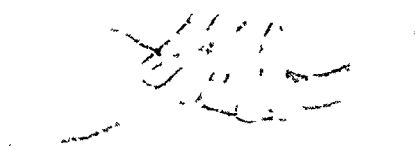
Basal ration only.

Fig. 24.

Basal ration + 8 c.c.
  antiscorbutic.
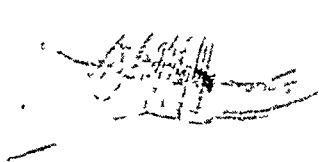
Basal ration + 5 c.c.
  antiscorbutic.

Basal ration + 2 c.c.
  antiscorbutic.
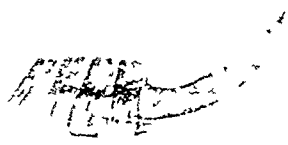
Basal ration only.
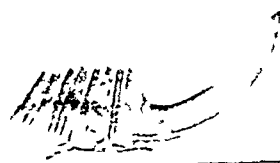
Fig. 25.

Figs. 24 and 25. Radiographs of the mandibles of guinea-pigs maintained on adequate diets and various deficiencies of antiscorbutic, illustrating that bone growth of the jaws and alveolar ridges is directly proportional to the vitamin C intake.

Figs. 17 and 21 (Plates V and VI) are photomicrographs of sections from the group of animals maintained on the basal ration only, i.e. with no orange juice supplement. It was found that the lactating mothers could not survive longer than 24 days. The young animals on the other hand, when maintained on the mother's milk during the lactation period and thereafter on the basal ration alone, survived in some instances as long as 39 days.

In view of the fact that the young survive longer than the mother with a complete absence of vitamin C in the diet, we might reasonably postulate the hypothesis that the young guinea-pig has some inherent resistance due, as in the rat, to the ability in some small degree to synthesize ascorbic acid or some precursor of vitamin C, such as hexuronic acid in the supra-renals or other tissues, although this ability is not possessed by the older animals. Alternatively, it may be due to a superior intensity of assimilation on the part of the embryonic tissue during gestation and a greater power of storage of vitamin C by the young animals carried over into the lactation period. Whatever the explanation of this phenomenon it is obvious from photomicrographs of the teeth that it operates only to a limited degree.

From a study of the photomicrographs here presented and truly representative of a very much larger number of sections examined, it is apparent that the microscopic tooth picture of both the lactating mothers and their young can be altered at will by varying the amounts of vitamin C in the diet of the mother. Further, if the vitamin C potency of the mother's milk is insufficient through faulty diet, dental dystrophies may be prevented in the young by the addition of suitable amounts of antiscorbutic as a supplement to the milk of the mother.

That the supporting structures of the teeth including the alveolar bone undergo degeneration in addition to the odontoblasts, ameloblasts and general connective tissue of the dental pulp is evident from the loosening of the molars in conditions of hypovitaminosis-C.

Scurvy is a disease in which the calcification process becomes abnormal. The bones are affected as well as the teeth and decalcification may take place.

Throughout these experiments it was noted that the legs of scorbutic guinea-pigs are apt to break spontaneously, and will not unite even if properly splinted, owing to the inability of the animal to form a satisfactory callus. Fig. 22 (Plate VII) shows a radiograph of a leg fracture which occurred in a scorbutic guinea-pig, and Fig. 23 shows the fracture united by a firm callus following the administration of curative doses of antiscorbutic in the form of orange juice, despite the fact that no attempt was made to set the limb.

The effect of hypovitaminosis-C on the jaw and facial bones is equally marked. In mature animals it was seen that the alveolar bone becomes thin and porous and the teeth loose and irregular with a clinical condition closely simulating chronic suppurative periodontitis and with typical pocket and pus formation.

During the developmental period similar degenerative changes occur and it is found that bone growth of the jaws and alveolar ridges was directly proportional to the vitamin C intake. This is well illustrated in Figs. 24 and 25 (Plate VIII), which show the relative growth of the jaws of animals on varying amounts of antiscorbutic.

Scurvy appears to be a capillary disease (Findlay, 1921), the essential pathological change in scurvy experimentally produced being the swelling and degeneration of the capillary endothelium as a result of which the flow of blood through the capillaries is retarded and severe congestion follows. This endothelial degeneration results in damage to the intercellular substance, and œdema and hæmorrhage occur. Stagnation of the blood results in deficient oxygenation of the tissues.

Although the processes are not yet fully understood it is obvious from these studies that vitamin C plays a most important rôle in general tissue metabolism and must be of the utmost importance to the health of all body-tissues.

In relation to the health of the periodontal structures, Boyle (1938) emphasizes what has frequently been observed, namely, that vitamin C deficiency is the only nutritional deficiency which produces the characteristic features of the systemic type of pyorrhœa in experimental animals. The very high incidence of gingivitis and other periodontal diseases observed in deficient or famine areas such as Ajmer-Merwara and Hissar (Day, 1944) where numerous cases of scurvy have been reported may conceivably have its origin at least in part in a condition of hypovitaminosis-C.

SUMMARY AND CONCLUSIONS.

Following the preliminary investigation of latent scurvy and its relation to dental defects, and of the effects of antiscorbutic deficiency upon the pregnant organism, this experimental work was undertaken with the object of investigating the effect of relative and absolute hypovitaminosis-C upon lactating mothers and their young.

As in the earlier work particular attention was given to the effect on the dental tissues, but the opportunity was also taken to study the reaction to this specific deficiency on the body-organism as a whole.

Our results leave no room for doubt that the specific needs of the organism for the antiscorbutic vitamin are equally as great during the period of lactation as during the gestation period.

Growth curves indicate that the average and percentage weight loss during the experimental period of 20 days' lactation was inversely proportional to the amount of antiscorbutic received by the mothers. As the only variable was the antiscorbutic potency of the mother's milk it may, therefore, be assumed that vitamin C was secreted into the mother's milk in approximately the same ratio in which it was administered to her by mouth.

Although, as might be expected, there was considerable individual variation, it was found that the average incisor growth per day in both the upper and lower jaw was directly proportional to the amount of antiscorbutic administered. The average growth of the lower incisors was in all cases greater than that of the uppers. The mean average growth per day was, therefore, also proportional to the vitamin C intake.

These remarkably constant results would, therefore, appear to confirm the observation that the rate of growth of the incisor teeth of guinea-pigs is a precise indication of the degree of scurvy. It is, therefore, reasonable to assume a specific response of the teeth to the presence or absence of the antiscorbutic vitamin.

The incisor growth rate was less in lactating animals than non-lactating animals of the same weight. This would seem to indicate an intensified effect of a lack of vitamin C during lactation.

It is considered that figures showing the percentage of total minerals in the bones and teeth are not of particular significance in view of the short experimental period, and for other reasons advanced it is thought 'that over this short period more importance attaches to the qualitative changes than to quantitative changes in the mineralization of the bones and teeth.

A study of the photomicrographs from transverse sections through the mandible and incisor teeth clearly demonstrates the profound effect of hypovitaminosis-C on the dental tissues and illustrates the importance of an adequate maternal diet during the period of lactation not only for the protection of the mother, but also to ensure adequate amounts of vitamin C in the mother's milk for effective protection of the young. Although the mother's diet may be only slightly deficient in vitamin content the antiscorbutic potency of the milk is proportionally reduced resulting in marked changes in the dental tissues of the young.

These pathological tissue changes run parallel to the other observed phenomena of interference with body-growth and tooth-growth and occur even in the absence of scurvy clinically recognizable as such.

It is apparent that the microscopic tooth picture of both the lactating mothers and their young can be altered at will by varying the amounts of antiscorbutic in the mother's diet; further, if the vitamin C potency of the mother's milk is insufficient through faulty diet, dental dystrophies and other degenerative changes may be prevented by the addition of suitable amounts of vitamin C as a supplement to the milk of the mother.

The effect of hypovitaminosis-C on the jaws and facial bones is very marked. In mature animals the alveolar bone becomes thin and porous and the teeth loose and irregular due to lack of support by the periodontal structures.

During the developmental period similar degenerative changes occur. Radiographs show that the growth of the jaws and alveolar ridges is directly proportional to the vitamin C intake.

During vitamin C deficiency characteristic features of the systemic type of gingivitis and pyorrhœa occur in experimental animals. In this connection it is of interest to note that a very high incidence of gingivitis has been observed in the famine district of Hissar where numerous cases of florid scurvy have been clinically recognized.

In the light of these studies it is considered that much of the degeneration of the oral tissues and bodily ill health has its origin in specific vitamin deficiencies and that scurvy of the latent or sub-acute type is much more common than is generally supposed.

In countries such as India where the dietaries of a large proportion of the population in many areas are greatly restricted through ignorance, poverty or climatic conditions, the danger of specific deficiencies is very great. Recent investigations undertaken and health measures adopted in the deficient and famine

areas of the Punjab have clearly shown the futility of improving the quantity of the diet unless the specific deficiencies are at the same time eliminated.

Despite the pronounced degenerative changes which have been shown to occur in the dentine and enamel of the teeth, there is no evidence to suggest that these changes have any relation to the process of dental caries.

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A DENTAL SURVEY IN AJMER-MERWARA.

BY

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AJMER-MERWARA is a separately constituted province in Rajputana with a total area of 1,562,106 acres. The population according to the Census of 1941 was 583,693.

The situation of the province is such that it is beyond the influence of the north-west and south-east monsoons. Rainfall is very uncertain and the predominantly agricultural population has constantly been faced with difficult economic conditions and food shortage. There are records of frequent famines from as early as 1661 to 1934-40, and scarcity of grass, grain and water has been an even more frequent occurrence.

In 1946, at the request of the provincial authorities, the writer carried out a diet and nutritional survey to study the nutrition problem in the province (Shourie, 1946). The following are some of the salient points arising out of those surveys :—

1. Comparison of the total requirements of different foodstuffs necessary to ensure a balanced diet for the population with the statistics of food production of the province shows that Ajmer-Merwara is a deficit province with respect to all foodstuffs.

2. Comparison of the cost of a balanced diet for a month with the monthly family income also indicates that the major part of the population cannot afford an adequate balanced diet.

3. Diet surveys carried out in the province show that (*a*) with respect to caloric value the diets consumed were on the whole satisfactory, and that most of the calories were derived from cereals. (*b*) Total protein consumption appears to be adequate but the proportion of animal protein is inadequate. (*c*) Calcium intake of most of the groups studied was below the recommended standards.

(*d*) Vitamin A consumption in all the groups studied is very inadequate. (*e*) Vitamins B_1 and B_2 group contents of the diets are adequate, as the diets are based on whole wheat and pulses. (*f*) Both the urban and rural diets were very deficient in their vitamin C content as very little raw uncooked vegetables or fruits were consumed.

4. Mortality and morbidity : (*a*) More than 20 per cent of the infants die before the age of one year and the infant mortality rate is not declining. (*b*) The mortality rate per thousand for males and females range between 0·7 to 6·84 and 0·9 to 5·91 respectively. The general experience of those concerned with infant welfare and maternity work supports the view that much sickness and mortality among infants and mothers are due to faulty feeding of both mother and child. (*c*) The annual report of the Chief Medical Officer, Ajmer-Merwara, shows that the diseases responsible for most of the sickness are malaria, diseases of the digestive system, and eye diseases. Nutrition workers have laid emphasis on the fact that malnourishment directly or indirectly affects the incidence of the above-mentioned diseases. (*d*) Nutritional surveys carried out in the area showed : (*i*) 4 to 6 per cent of the children examined suffered from diet-deficiency diseases, and (*ii*) anæmias were very common amongst females during the reproductive period. (*e*) The population of the province is increasing at a very rapid rate, and if the resources of the province are not properly supplemented, malnourishment will increase to a still greater extent.

In view of (*i*) the peculiar situation of the province, (*ii*) the fact that the population has suffered through famines and scarcities, and (*iii*) lack of data regarding the incidence of dental and paradontal diseases, a survey of the incidence of diseases of the teeth and gums was carried out in Ajmer-Merwara.

METHODS OF INVESTIGATION AND RECORDING.

Dental caries.—A detailed dental inspection in which all the available tooth surfaces in the mouth were examined using a dental mirror and probe was made in the case of each child. The total number of teeth and the number of deciduous and permanent teeth were recorded. All extracted teeth were recorded as carious except when known to have been lost as a result of trauma, pyorrhœa or extracted for some other reason. In recording the extent of caries the classification of Day and Sedwick (1934) was employed.

Presence of deciduous and permanent teeth.—The alphabetical and numerical system for charting the respective deciduous and permanent teeth was used.

In addition, details regarding sex, age and residence (rural or urban) were noted.

Dental fluorosis or mottled enamel.—Presence or absence of mottled enamel was recorded and Dean's (1936) system of classification of mottled enamel was used.

According to Dean the seven classes are : (1) normal, (2) questionable, (3) very mild, (4) mild, (5) moderate, (6) moderately severe, and (7) severe. Brown stain begins at the 'moderate' stage.

The following groups were studied :—

Group.	Rural or urban.	Name of institution.	Number of persons examined.
1	Rural (children) ...	Primary School for boys (Gobind Garh).	281
2	,, ,, ...	Middle School for boys (Pisangan).	
3	,, ,, ...	Government Girls' School (Pisangan).	53
4	Urban (children) ...	Government High School for boys (Ajmer).	387
5	,, ,, ...	Municipal Schools for boys (Ajmer).	
6	,, ,, ...	Mayo College for boys (Ajmer).	145
7	Urban (adults) ...	Longia Hospital for females (Ajmer).	129
8	,, ,, ...	Maternity Home for females (Ajmer).	
9	Rural (adults) ...	B. B. & C. I. Rly. Workshops, males (Ajmer).	161
		TOTAL ...	1,156

RESULTS OF INVESTIGATION.

In Table I is given the incidence of dental caries amongst school children in Ajmer-Merwara. In Table II is recorded the percentage of freedom from caries, number of cavities per child and average caries figure for various comparable groups examined in India and the United States of America. Table III compares dental caries in rural and urban children. Table IV shows incidence of dental caries in the Mayo College students. Table V gives figures for caries in adults in Ajmer-Merwara.

DISCUSSION.

Table I shows that 59·2 per cent of the children in Ajmer-Merwara were free from caries. In similar surveys carried out in other parts of India (Shourie, 1912, 1916) freedom from caries was found to be 44·5 and 39·8 per cent. The percentage of freedom from caries in the present group is higher than previously met with,

TABLE I(a).

Dental caries in children in Ajmer-Merwara province.

Age in years.	Number of children.	Percentage free from caries.	DECIDUOUS TEETH.		PERMANENT TEETH.		All teeth percentage carious.
			Number.	Percentage carious.	Number.	Percentage carious.	
5	4	50·0	70	8·5	14	0·0	7·1
6	15	53·3	263	12·1	89	3·3	9·9
7	46	58·7	696	11·2	412	0·2	7·1
8	54	55·5	733	8·3	566	1·6	5·1
9	87	59·7	1,089	7·8	1,020	1·2	4·6
10	111	54·9	1,107	9·3	1,611	1·1	4·8
11	97	59·5	563	15·2	1,931	1·5	4·5
12	86	60·4	376	9·5	1,850	2·2	3·5
13	61	73·7	96	8·3	1,571	1·6	1·9
14	47	72·3	64	9·3	1,132	1·6	2·5
15	37	64·0	25	16·0	1,007	3·3	3·6
16	15	80·0	0	0·0	399	1·5	1·5
17	2	100·0	0	0·0	56	0·0	0·0
18	6	83·3	0	0·0	171	0·6	0·6
TOTAL	668	59·2	...	...	...	...	...

TABLE I(b).

	Total number of teeth.	Percentage of teeth carious.	EXTENT OF CARIES.				Average caries figure.
			1	2	3	4	
Deciduous ...	5,082	10·1	110	232	61	103	0·23
Permanent ...	11,829	1·6	162	21	6	8	0·021

although Day (1944) found 74·2 per cent of children free from dental decay in Hissar (Punjab). In that survey a group of children of an average age of approximately 12 years was examined. In Table II figures obtained in the

present investigation are compared with those arrived at by Day (1940, 1944) and Day and Sedwick (*loc. cit.*) in various investigations in India and in the U.S.A.

TABLE II.

Comparison of groups in the U.S.A. and India.

Group.	Names of investigators.	Number of children.	Average age.	Children caries free, per cent.	Number of cavities per child.	Caries index.
Rochester (U.S.A.) ...	Day and Sedwick (1934)	433	12·94	0·2	21·76	0·83
Lahore (India) ...	Day and Tandan (1940)	756	13·58	5·95	5·74	0·21
Hissar (India) ...	Day (1944)	314	11·87	74·2	0·62	0·02
Ajmer-Merwara (India)	Shourie (1947) (present investigation).	244	11·85	63·5	0·91	0·06

It is interesting to note that the Ajmer-Merwara group shows less freedom from caries, a greater number of cavities per child, and higher average caries figures than the Hissar group, and quite the opposite compared with the other two groups. It may be added that Hissar district like Ajmer-Merwara province is also a famine-stricken area. Day conducted a study of dental and paradontal diseases during the second year of acute shortage of food when famine was declared. The difference in incidence of caries may be accounted for by the fact that the population examined in Hissar (Punjab) was more rural than that in Ajmer-Merwara.

The low incidence of dental caries in famine-stricken areas fails to accord with the theories purporting to explain caries in terms of dietary factors. According to modern standards of nutrition the diets consumed in Ajmer-Merwara are, even in years when there is no famine, unbalanced and during times of scarcity would be much more so. The population of Ajmer-Merwara suffered from food shortage for a period of two years or more (1938–40) and, if not all, most of the children examined must have lived under famine or sub-famine conditions for considerable periods. If the dental caries process is directly affected by quantitative or qualitative nutritional deficiencies a reasonable expectation would have been a much higher caries incidence in the groups examined in the present investigation.

The Hissar (Punjab) and Ajmer-Merwara districts have certain features in common. The population in both districts is subsisting on very ill-balanced diets and both are potentially famine areas with periods of famine regularly recorded. Diet surveys in both districts show that the diets are preponderating in cereals and are inadequate in animal proteins, calcium, vitamin A and ascorbic acid. The somewhat higher caries incidence in the Ajmer-Merwara group may be explained by the fact that it is more urban than the Hissar group. Despite this variation in caries incidence both groups show an extremely low susceptibility to dental caries on the basis of comparison with other countries and even with other districts

in India. These considerations lend support to the view of Day (1940) that ' a diet nutritionally sound according to our modern concept has little if any bearing on caries immunity '.

Furthermore, both Ajmer-Merwara and Hissar (Punjab) are endemic fluorosis areas. In the present, Ajmer-Merwara investigation data was also collected regarding the incidence and degree of mottled enamel and the results show that 61 per cent of the persons examined showed definite mottling.

In earlier investigations in this country (Day, 1940; Shourie, 1946) a lower incidence of dental decay was recorded in children showing mottled enamel than in those showing no mottling of the teeth.

The very high freedom from dental caries met with in the group under study may be due in part to local or general effects of fluorine ingestion and in part to the physical nature of diet.

In Table III a comparison is made of the incidence of dental caries in rural and urban children in Ajmer-Merwara. There is no marked difference in the two groups though the percentage of total teeth carious and average caries figure show some advantage for the rural group. The differences in the two groups are not as clear cut as were found in rural and urban children in the Delhi province.

TABLE III(a).

Dental caries in rural and urban children, Ajmer-Merwara.

Age in years.	Rural or urban.	Number of children.	Percentage free from caries.	DECIDUOUS TEETH.		PERMANENT TEETH.		All teeth percentage carious.
				Number.	Percentage carious.	Number.	Percentage carious.	
5	R	4	50·0	70	8·5	14	0·0	8·5
	U	...	...	...	...	...	...	...
6	R	8	50·0	127	20·4	59	3·3	15·0
	U	7	57·1	136	4·4	30	3·3	4·1
7	R	21	66·6	321	8·0	183	0·6	5·3
	U	25	42·0	375	13·8	229	0·0	8·6
8	R	19	42·1	254	11·0	203	1·4	6·7
	U	35	62·8	479	6·8	363	0·0	4·2
9	R	45	62·2	575	6·6	513	1·9	4·4
	U	42	57·1	514	9·1	507	0·0	4·8
10	R	47	53·1	470	11·9	693	0·9	5·2
	U	64	56·2	637	7·5	918	1·4	3·9

TABLE III(*a*)—*concld.*

Age in years.	Rural or urban.	Number of children.	Percentage free from caries.	DECIDUOUS TEETH.		PERMANENT TEETH.		All teeth percentage carious.
				Number.	Percentage carious.	Number.	Percentage carious.	
11	R	39	53·8	322	13·9	663	1·9	5·6
	U	58	63·7	241	16·5	1,268	1·4	3·9
12	R	29	75·9	161	3·1	593	1·8	2·1
	U	57	52·6	215	14·4	1,257	2·4	4·2
13	R	18	83·3	50	2·0	441	1·3	1·2
	U	43	69·7	46	4·7	1,130	1·6	6·4
14	R	19	78·9	32	0·0	486	1·0	0·9
	U	28	67·8	32	15·6	646	2·1	2·8
15	R	21	61·9	21	14·1	562	3·2	3·0
	U	16	43·7	4	25·0	445	3·5	3·8
16	R	6	66·6	0	0·0	168	2·9	2·9
	U	9	88·8	0	0·0	231	0·5	0·4
17	R	...	...	...	...	...	...	...
	U	2	100·0	0	0·0	56	0·0	0·0
18	R	5	80·0	0	0·0	143	0·7	0·7
	U	1	100·0	0	0·0	28	0·0	0·0

TABLE III(*b*).

			Total number of teeth.	Percentage of teeth carious.	EXTENT OF CARIES.				Average caries figure.
					1	2	3	4	
Deciduous	...	R	2,403	9·7	52	123	16	44	0·21
		U	2,679	10·1	58	109	45	59	0·24
Permanent	...	R	4,721	1·6	70	3	4	1	0·019
		U	7,108	1·7	92	18	2	7	0·022

In Table IV is given the incidence of dental caries in a group of children of families of very high social status and of Indian princes. In the following figures

TABLE IV(a).

Dental caries incidence in Mayo College students, Ajmer city.

Age groups in years.	Number of persons.	Percentage free from caries.	DECIDUOUS TEETH.		PERMANENT TEETH.		All teeth percentage carious.
			Number.	Percentage carious.	Number.	Percentage carious.	
5 to 9	23	56·0	310	6·4	246	4·0	5·4
10 to 15	64	56·2	193	12·2	1,494	2·6	3·3
16 to 20	45	51·1	15	...	1,298	4·2	5·3
21 to 30	10	50·0	...	...	298	11·6	3·6

TABLE IV(b).

	Total number of teeth.	Percentage of teeth carious.	EXTENT OF CARIES.				Average caries figure.
			1	2	3	4	
Deciduous ...	518	7·1	12	12	5	8	0·16
Permanent ...	3,336	3·5	59	36	9	13	0·062

for the 10 to 15-year age group are compared with the figures of similar age groups from Ajmer-Merwara :—

Name of institution.	Number of children.	Age.	Children free from caries, percentage.	Number of cavities.	Average caries figure.
Mayo College (Ajmer)	64	10 to 15	56·2	0·91	0·065
Other schools (Ajmer-Merwara) ...	439	10 to 15	61·5	0·95 ·	0·07

The results show that though freedom from caries is greater in children of the lower economic group the number of cavities per child and the average caries figure is a little lower in the better-class group. This observation lends support to the view that 'economic status has little influence on the development of dental caries' (Shourie, 1941).

In Table V is recorded the incidence of dental caries in adults in Ajmer-Merwara. The degree of freedom ranges between 35·4 to 100 per cent in the various age groups. It is of interest to compare these figures with results of the examination of adult groups in other countries. Clinical dental examination of 1,047 naval officers and enlisted men of the United States Navy by Schlack (1940) indicates that the prevalence of past and present caries observed on initial examination is of the order of 24 permanent tooth areas per man. Klein (1941) records that of 642 military recruits aged from 21 to 35 years, only 97, i.e. 15·2 per cent, satisfied the dental requirements for full military service and the selected group showed that per man 0·7 teeth were sufficiently carious to require extraction, 7·1 tooth surfaces required filling, 2·5 teeth had already been extracted, 0·7 teeth required extraction and 9 out of every 10 men required partial dentures or bridges; corresponding figures for the remaining 545 men were 2·4, 7·6, 13·0, 2·4, and 9 out of every 10 men required full or partial dentures.

TABLE V(*a*).

Incidence of dental caries in adults in Ajmer-Merwara.

Age groups in years.	Sex.	Number of persons.	Percentage free of caries.	DECIDUOUS TEETH.		PERMANENT TEETH.		All teeth percentage carious.
				Number.	Percentage carious.	Number.	Percentage carious.	
16 to 20	F	28	39·3	2	...	821	1·9	1·9
	M	11	35·4	...	...	335	3·2	3·2
21 to 30	F	67	64·1	3	...	785	4·7	4·7
	M	43	72·1	...	...	1,359	1·4	1·4
31 to 40	F	30	56·6	...	...	940	3·0	3·0
	M	60	76·6	...	...	1,889	1·4	1·4
41 to 50	F	3	66·6	...	...	96	0·0	0·0
	M	37	83·8	...	...	1,168	0·7	0·7
51 to 60	F	1	100·0	...	...	32	0·0	0·0
	M	10	100·0	...	...	316	0·0	0·0

TABLE V(b).

	Sex.	Total number of teeth.	Percentage of teeth carious.	Extent of caries.				Average caries figure.
				1	2	3	4	
Deciduous ...	F	5	...	...	...	...	...	...
	M	...	...	...	...	...	...	...
Permanent ...	F	2,674	3·1	34	8	4	37	0·078
	M	5,067	1·3	24	15	7	21	0·031

Miller (1943) in a preliminary survey of the incidence of dental caries in Great Britain published statistics regarding the average number of teeth missing, requiring extraction, requiring filling, filled and sound, of British army recruits at enlistment and these figures were tabulated according to age. The groups ranged in age from 18 to 30 years and each contained over 100 men. In the following are given values per mouth for the 18- and 30-year age groups :—

Age.	Teeth missing.	Requiring extraction.	Requiring filling.	Filled.	Sound.
18	3·7	1·2	2·3	0·6	24·2
30	11·1	4·8	2·1	0·4	13·6

A comparison of figures obtained in this investigation with those recorded above show that Indian adults like Indian children show greater freedom from dental decay than individuals of similar age in the U.S.A. and in Great Britain.

In Table V is given the incidence of dental decay in adult females and males. In this case a fair comparison cannot be made as the males and females belonged to two entirely different groups. The females were mostly from urban families, while the males who were workers in the Loco Workshops came from rural areas. The significantly lower incidence in the male group is probably a geographical rather than a sex variation.

SUMMARY.

In conjunction with a diet and nutritional survey to study the nutrition problem in the province of Ajmer-Merwara an oral survey has been carried out for the purpose of obtaining statistics regarding dental and oral diseases.

Despite the ill-balanced nature of the diet. the preponderance of cereals. inadequacy of animal protein. calcium, vitamin A and ascorbic acid. and high mortality and morbidity figures. the results of this investigation support the opinion reached following a similar investigation in Hissar (Punjab) that 'a diet nutritionally sound according to our modern concept has little. if any. bearing on caries immunity'.

The finding that 59·2 per cent of the children examined were free from caries fails to accord with the theories purporting to explain caries in terms of dietary factors. The possibility of fluorine being an influencing factor in the high degree of immunity reported is also discussed.

Figures are given for caries incidence in rural and urban groups, in groups of different social status and in adults in comparison with groups of other countries.

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EFFECTS OF HEPARIN ON THE ACTION OF THE VENOMS OF SOME AMERICAN VIPERINE SNAKES.

BY

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AND

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It has recently been reported by Ahuja *et al.* (1946*a*, 1946*b*) that heparin effectively neutralizes, *in vitro* and *in vivo*, the blood coagulant action of the venom of *Vipera russellii* (daboia) and to a somewhat lesser extent that of *Echis carinatus.*

The present communication reports the results of experiments on the effects of heparin on the action of the venoms* of the species of viperine snakes listed in Table I, together with the coagulant activity of their venoms on whole blood.

The heparin† which was used in the present experiments consisted of the sodium salt dissolved in physiological saline solution in the proportion of 10 mg. per c.c. and representing approximately 110 Toronto Units.

ACTION OF HEPARIN ON THE BLOOD COAGULANT ACTIVITY OF CERTAIN VENOMS *in vitro.*

It will be seen from Table I that of the seven venoms tested moccasin venom inhibited clotting, while the venoms of *B. alternatus, B. jararacussu, B. atrox, B. cotiara, B. neuwiedii* and *Crotalus terrificus* were markedly blood coagulant. In the case of the venoms from the six latter snakes heparin effectively counteracted the coagulant action either by completely preventing clotting for the 24-hour

TABLE I.

Clotting time of 1 c.c. of whole blood (sheep) in the presence of the venom of :

Venom in mg.	B. alternatus.		B. jararacussu.		B. atrox.		B. cotiara.		B. neuwiedii.		Crotalus terrificus.		Moccasin venom.	
	Without heparin.	With heparin, 1 mg.	Without heparin.	With heparin, 1 mg.	Without heparin.	With heparin, 1 mg.	Without heparin.	With heparin, 1 mg.	Without heparin.	With heparin, 1 mg.	Without heparin.	With heparin, 1 mg.	Without heparin.	With heparin.
	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	min. sec.	
0·1	1 2	2 45	1 ..	1 40	.. 40	.. 40	1 2	2 6	1 30	1 30	.. 55	1 5	No clot 24 hrs.	..
0·01	3 10	23 ..	1 40	11 30	1 45	1 52	4 42	25 ..	3 20	17 30	2 27	4 12	12 35	..
0·001	5 10	No clot 5 hrs. ; clot 21 hrs.	5 ..	3 hrs.	3 20	24 hrs.	8 10	No clot 5 hrs.; clot 21 hrs.	7 ..	3 hrs.	2 58	55 ..	8 ..	..
0·0001	8 4	No clot 24 hrs.	5 5	No clot 24 hrs.	7 4	No clot 24 hrs.	8 30	No clot 24 hrs.	7 5	No clot 24 hrs.	3 44	No clot 5 hrs.; clot 21 hrs.	7 52	..
Sheep blood control.	6 ..	No clot	7 20	No clot	8 23	No clot	9 ..	No clot	7 20	No clot	6 ..	No clot	6 ..	..

period of observation or by prolonging the clotting time from a matter of few minutes to that of hours, depending on the concentration of venom used. It will be seen for instance that (*a*) 1 mg. of heparin in the presence of 0·01 mg. of *B. alternatus* venom prolonged the clotting time of blood from 3 minutes 10 seconds to 23 minutes, which is nearly four times the normal clotting time of sheep blood and (*b*) 1 mg. of heparin failed to prevent the coagulant action of 0·001 mg. of this venom although it prolonged the clotting time from 5 minutes 10 seconds to over 5 hours. Similar results were obtained with the venoms of the other five viperine snakes included in Table I.

The addition of oxalate or citrate to the blood before the addition of venom did not significantly alter the coagulant action of these venoms although the clotting time was comparatively increased.

ACTION OF HEPARIN ON THE TOXICITY OF CERTAIN VIPERINE VENOMS *in vivo*.

It is evident from the results recorded in Table II that (*a*) 20 mg. of heparin counteracted the toxic effects of 5 mg. of *B. alternatus* venom, representing at least 20 intravenous minimum lethal doses, and that half this dose of heparin rendered inert at least 33 m.l.d. of the venom of *B. atrox*, 10 m.l.d. of the venom of *B. jararacussu* and 5 m.l.d. of the venom of *B. cotiara*; (*b*) when the quantity of venom injected was increased beyond certain limits heparin apparently failed to counteract the toxic effects of such a heavy dose; and (*c*) heparin failed to neutralize, *in vivo*, the venoms of *B. neuwiedii* and *Crotalus terrificus*.

TABLE II.

Venom was mixed with heparin, incubated at 37°C. for 10 minutes, and the mixture given intravenously to rabbits weighing about 1,500 g. to 2,000 g.

Snake venom.	Dose in mg.	Number of lethal doses injected.	Heparin in mg.	Result.
	0·05	0·2	Nil	Survived.
	0·10	0·4	Nil	,,
	0·20	0·8	Nil	,,
	0·25	1·0	Nil	Died, 10 min.
B. alternatus ...	0·50	2·0	Nil	Died, 3 min.
	0·50	2·0	10·0	Survived.
	5·00	20·0	20·0	,,
	7·50	30·0	20·0	Died, 15 min.
	10·00	40·0	20·0	Died, 3 min.

TABLE II—*contd.*

Snake venom.	Dose in mg.	Number of lethal doses injected.	Heparin in mg.	Result.
B. jararacussu ...	0·01	0·1	*Nil*	Survived.
	0·02	0·2	*Nil*	,,
	0·05	0·5	*Nil*	,,
	0·10	1·0	*Nil*	Died, 4 min.
	0·50	5·0	*Nil*	Died, 2 min.
	0·50	5·0	10·0	Survived.
	0·50	5·0	10·0	,,
	1·00	10·0	10·0	,,
	1·00	10·0	10·0	,,
	2·00	20·0	10·0	Died, 3 min.
	2·00	20·0	20·0	Died, 2 hrs.
B. atrox ...	0·05	0·33	*Nil*	Survived.
	0·10	0·66	*Nil*	Died, 3 days.
	0·15	1·00	*Nil*	Died, 6 min.
	0·20	1·33	*Nil*	Died, 3 min.
	0·50	3·33	*Nil*	Died, 3 min.
	0·50	3·33	5·0	Survived.
	1·00	6·66	10·0	,,
	2·00	13·33	10·0	,,
	5·00	33·33	10·0	,,
	6·00	40·00	30·0	Died, 3 min.
	7·50	50·00	20·0	Died, 6 min.
	10·00	66·23	20·0	Died, 3 min.

- TABLE II—*concld.*

Snake venom.	Dose in mg.	Number of lethal doses injected.	Heparin in mg.	Result.
B. cotiara	0·5	0·5	Nil	Survived.
	1·0	1·0	Nil	Died, 2 min.
	1·0	1·0	10·0	Survived.
	5·0	5·0	10·0	,,
	10·0	10·0	20·0	Died, 2 min.
B. neuwiedii	0·5	...	Nil	Died, 3 min.
	0·5	...	10·0	Died, 29 hrs.
Crotalus terrificus	0·5	...	Nil	Died, 21 hrs.
	1·0	...	10·0	Died, 6 hrs.
	2·0	...	10·0	Died, 2 hrs. 40 min.

DISCUSSION.

In these experiments no attempt has been made to duplicate the natural mode of injection of venom as it was not possible to determine the m.l.d. of the viperine venoms tested with any degree of accuracy when administered by the intramuscular or subcutaneous routes. The absorption of venoms by these routes is so irregular and variable that it is difficult to draw accurate conclusions from such experiments. Anderson (1932) found the same difficulty with daboia venom and, for this reason, he adopted the intravenous route in the standardization of antivenene. Our experiments were carried out with freshly prepared solutions in normal saline of dried venom given by the intravenous route. This was found to cause death in the control animals consistently without any great variation in the lethal dose.

It has been previously reported that one part by weight of heparin can effectively counteract *in vivo* the lethal action of an equivalent amount of Russell's viper venom, and that it is possible to protect a rabbit against 10 mg. of this venom (representing over 60 lethal doses) given intravenously. With the venom of *Echis carinatus* it was found that (a) a comparatively larger quantity of heparin was required to counteract the toxic effects of this venom under similar experimental

conditions *in vivo*, and (*b*) that when the dose of venom injected was increased beyond 20 lethal doses, heparin was of no therapeutic value. The venoms of *B. alternatus, B. jararacussu, B. atrox* and *B. cotiara* resemble the venom of *Echis carinatus* in these respects. By the use of heparin it is possible to protect experimental animals against a number of lethal doses of the above venoms, but only up to a limit, which varies with the type of venom used. Beyond this limit, toxic fractions other than blood coagulants, such as cardiotoxins, hæmorrhagins, etc., appear to exert a lethal action and heparin is ineffective against them. This view is supported by the observation that heparin is ineffective against the venom of *Crotalus terrificus*, which like that of the Australian tiger snake, *Notechis scutatus*, appears to contain, besides the blood coagulant fraction, a powerful neurotoxic fraction.

Since venoms of most viperine snakes contain species specific blood coagulant fractions which are intimately related to their toxicity, and since heparin has been shown to neutralize *in vitro* and *in vivo* the blood coagulant fractions of the venoms of a number of snakes belonging to the family *Viperidæ*, heparin would appear to be a rational physiological antidote to counteract the blood coagulant effects in viperine poisoning.

The evidence presented points to heparin as a useful remedy which has given promising results in the treatment of poisoning in experimental animals with venoms of certain American viperine snakes. It is not suggested that heparin can replace specific antivenom serum therapy. All that can be said at present is that the results reported are sufficiently encouraging to warrant the therapeutic trial of heparin in definitely known cases of bites by vipers of the above-mentioned species, when specific antivenene is not available, or becomes available only in limited quantity after a delay of several hours.

SUMMARY.

1. Experimental evidence is presented to show that heparin effectively neutralizes, *in vitro*, the blood coagulant action of the venoms of viperine snakes: *B. alternatus, B. jararacussu, B. atrox, B. cotiara, B. neuwiedii* and *Crotalus terrificus*.

2. Heparin counteracts some of the toxic effects of the venoms of *B. alternatus, B. jararacussu, B. atrox* and *B. cotiara* in experimental animals.

3. The trial of heparin as a therapeutic agent in the bites by the above-mentioned viperine snakes is suggested.

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NOTICE.

The following has been received for announcement.

—Editor, I.J.M.R.

20th June, 1947.

NAPT COLONIAL SCHOLARSHIPS, 1947.

THE award of two Scholarships in 1946 proved so satisfactory that the NAPT has decided to offer six Scholarships this year. These will be open to doctors and other medical personnel throughout the British Colonial Empire. The successful holders will come to Britain for a period of six months or longer to study tuberculosis in its widest aspects, clinical, administrative and social.

2. The award will be divided as follows :—

(a) Two Scholarships (value £120 each) to registered doctors in the Colonial Medical Service.

(b) Two Scholarships (value £100 each) to medical graduates of native Medical Schools in the British Colonies.

(c) Two Scholarships (value £80 each) to Matrons, Nurses, Health Visitors, or other members of Colonial Sanitary Departments.

3. The successful candidates will be eligible for lodging and training allowances from Colonial Government funds, and the details of their training during the tenure of the Scholarship will be supervised by the NAPT. Travelling expenses, purchase of books and other incidental expenditure will fall to be met by scholars out of their Scholarship moneys.

4. Applications should be made through the Colonial Medical Department, who will forward them to the Colonial Office, which will make recommendations to the Council of the NAPT.

5. The NAPT believes that these awards will further health education in its broadest sense, and encourage the formation of anti-tuberculosis services throughout the different Colonial territories.

6. The Scholarships will be held by young men or women who wish to specialize in tuberculosis work, either fully or part-time, and who will then carry the knowledge they have gained into the various Colonial territories where they will serve.

7. An announcement of the method of sending in applications will be made shortly by the Colonial Office.

May, 1947.

NOTICE.

The following has been received for announcement.

—Editor, I.J.M.R.

20th June, 1947.

LEISHMAN, ALEXANDER AND PARKES MEMORIAL PRIZE FUND.

It is announced that the following prizes are available for award in 1948. Conditions of award are as stated.

Prize.	Open to	Remarks.
1. The Leishman Memorial Prize (silver-gilt medal and approx. £30).	Officers R.A.M.C. or Officers removed from the Corps but still on the Active List. Officers R.A.D. Corps. (Officers must be serving on Regular or Short Service Commissions.)	Awarded for the best work in any branch of Medicine, Surgery or the allied sciences, or in connection with the general duties of the R.A.M.C. or R.A.D. Corps, brought to the notice of the R.A.M.C. Prize Funds Committee during the year, but not necessarily completed within the year.
2. The Alexander Memorial Prize (silver-gilt medal and approx. £70).	R.A.M.C. Officers as above.	Awarded to the Officer who by professional work of outstanding merit has done most to promote the study and improvement of Military Medicine, Military Surgery, Military Hygiene or Military Pathology during the year.

Prize.	Open to	Remarks.
3. The Parkes Memorial Prize (silver-gilt medal and approx. £60).	Regular serving Medical Officers on full pay of the Royal Navy, Army or the Indian Army.	First consideration will be given to original articles or reports of investigations of value from the point of view of Military Medicine, Surgery, Hygiene or Pathology, and published in one or other of the various Medical Journals. Part authorship of an article is not admissible.
		Awarded for professional work of outstanding merit which has done most to promote the study of Naval or Military Hygiene. First consideration will be given to articles or reports of investigations of value from the point of view of Naval or Military Hygiene published in one or other of the various Medical Journals. Part authorship of an article is not admissible.

Note :—The Alexander Memorial Prize and the Parkes Memorial Prize are not open to officers on the staffs of the Royal Naval Medical School, the Royal Army Medical College or the Army School of Hygiene.

Recommendations should be sent in through the usual channels with copies of original articles or reports of investigations, to reach the Hon. Secretary, R.A.M.C. Prize Funds Committee, R.A.M. College, Millbank, London, S.W.1, by 31st December, 1947.

The following prizes have been awarded for the year 1946 :—

Parkes Medal and £60.	Major-General F. Harris, C.B.E., M.C., M.B., late R.A.M.C.	For distinguished work in Hygiene in India and the Far East.
Leishman Medal and £30.	Lieut.-Colonel A. D. Young, D.S.O., M.B., R.A.M.C.	For a paper on 'The Parachute Field Ambulance'.
Alexander Medal and £70.	Lieut.-Colonel W. H. Hargreaves, M.R.C.P., R.A.M.C.	For his published work on Amœbiasis.

NOTICE.

The following has been received for announcement.

—Editor, I.J.M.R.

25th July, 1947.

THE CONSULTANTS PRIZE.

THE Consultants to the War Office and the Armies in the Field in the late war have presented a sum of money to the R.A.M.C. in order to found a Consultants Prize, to be competed for at intervals of one to three years.

This prize will be awarded for the first time in 1948 and will be to the value of 25 guineas. The prize is open to serving officers of the Royal Army Medical Corps, holding a regular or a short service commission.

The first prize will be awarded for an essay of not more than 10,000 words on a professional subject, based on the author's own experiences between 1939 and 1946. It is hoped that these essays will ensure that valuable war experience, which would otherwise be lost, will be recorded for future guidance and possibly for publication.

Entries should be sent in through the usual channels, so as to reach the Hon. Secretary, R.A.M.C. Prize Funds Committee, R. A. M. College, Millbank, London, S.W.1, not later than 1st August, 1948.

FURTHER OBSERVATIONS ON THE CULTIVATION OF RABIES VIRUS *IN VITRO*.

BY

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INTRODUCTION.

VEERARAGHAVAN (1946*a*) has presented evidence to show that rabies virus can multiply in a cell-free medium containing steamed sheep-brain extract, sheep-serum, glycine and peptone. Experiments were subsequently undertaken to determine whether the addition of accessory factors especially those belonging to the B group of vitamins or certain amino acids to the above medium had any beneficial effect on the multiplication of the virus. Details of the work are presented in this paper.

MATERIAL AND METHODS.

The method of cultivation of the virus *in vitro* was on the lines described by Veeraraghavan (1916*a*). Details regarding the preparation of the culture medium and the technique adopted are given below :—

Brain extract.—Normal sheep's brain was dissected out with aseptic precautions from freshly slaughtered sheep obtained from the local slaughter house. A portion of the brain was weighed and washed three or four times in sterile distilled water to get rid of blood. It was emulsified in a Waring blendor for six minutes with ice-cold distilled water to give a 10 per cent emulsion. The emulsion was centrifuged for two hours at 3,000 r.p.m. and the supernatant steamed in a steam sterilizer for half an hour on three consecutive days and, if found sterile, used in the preparation of the medium.

Serum.—Seitz-filtered sheep-serum was used.

Peptone.—A solution of 1·5 per cent Difco bacto-peptone in distilled water was used. The solution was sterilized by steaming for half an hour each day for three consecutive days.

Glycine.—A solution of 20 per cent glycine (B.D.H.) in distilled water was used. It was sterilized by steaming for half an hour each day for three consecutive days.

Tryptophan.—A solution containing 2 mg. of tryptophan per c.c. was prepared in sterile distilled water and sterilized by steaming for half an hour.

Accessory factors.—Pure thiamine hydrochloride, nicotinic acid, pyridoxine hydrochloride, calcium pantothenate and riboflavin were used. Separate solutions containing 1 mg. per c.c. of each of these factors were prepared in sterile distilled water and steamed for half an hour. The final concentration of each of the factors used in the culture medium was of the order of 2 μg. per c.c. A biotin concentrate obtained through the courtesy of Dr. (Miss) Kamala Bhagvat was used in these experiments. The strength of the preparation was not known. It was diluted 1 in 10 with sterile distilled water, sterilized by steaming for half an hour and 0·2 c.c. of the solution used for every 100 c.c. of the culture.

Virus inoculum.—Supernatant from a 20 per cent suspension of rabies fixed-virus sheep-brain was used for inoculating culture media. The virus suspension was prepared as follows: The brain of a sheep completely paralysed after subdural inoculation with the Paris strain of rabies fixed-virus was dissected out aseptically. A portion of the infected brain was weighed and washed in sterile distilled water to get rid of blood. It was emulsified in a Waring blendor for six minutes with ice-cold distilled water to give a 20 per cent emulsion. The emulsion was centrifuged for half an hour at 3,000 r.p.m. and the supernatant used as the inoculum.

Although it is desirable to use fresh fixed-virus sheep-brain for the preparation of the virus inoculum, in practice it was found inconvenient. In the experiments reported, fixed-virus sheep-brain stored in 50 per cent glycerine for a period of two or three days was used with good results.

Experimental animals.—Young healthy guinea-pigs of 200 g. to 250 g. weight were used as test animals.

Virus titration.—The virus content of a culture was determined by inoculating guinea-pigs subdurally with 0·2 c.c. of various dilutions of the culture in distilled water. The highest dilution which proved infective to at least 50 per cent of the inoculated guinea-pigs was determined. From this the virus content of the culture in terms of the minimum subdural lethal dose (m.l.d.) per c.c. was calculated by multiplying the highest infective dilution by five, since 0·2 c.c. was the inoculum used.

Sterility.—Sterile technique was used throughout and the cultures after incubation were regularly tested for aerobic and anaerobic sterility.

EXPERIMENTAL.

Effect of emulsifying normal and fixed-virus sheep-brain in a Waring blendor instead of grinding in a mortar.

It has been reported by Veeraraghavan (1946a) that a concentration of 2·5 million m.l.d. per c.c. of rabies virus can be obtained in cultures *in vitro* in a medium containing 1·5 per cent steamed sheep-brain extract, 2·5 per cent

TABLE I.

Fate of guinea-pigs inoculated subdurally with 0·2 c.c. of culture in dilutions.

Experiment No.	Flask	Number of hours at 37°C.	1/2,000	1/3,000	1/5,000	1/10,000	1/20,000	1/500,000	1/700,000	1/1 m.†	1/2 m.	1/5 m.
1	A	0	7/9*, 9/12	11/13, S	S, S	…	…	…	…	…	…	…
1	A	24	…	…	…	…	…	5/7, 6/8	S, S	S, S	…	…
1	B	0	…	…	5/7, 7/8	6/7, 8/10	S, S	…	…	…	…	…
1	B	24	…	…	…	…	…	6/7, 8/10	5/7, 7/9	5/7, 8/10	S, S	S, S
2	A	0	5/7, 8/10	8/10, S	S, S	…	…	…	…	…	…	…
2	A	24	…	…	…	…	…	6/7, 6/8	S, S	S, S	…	…
2	B	0	…	…	…	6/7, 9/11	S, S	…	…	…	…	…
2	B	24	…	…	…	…	…	6/8, 6/8	…	5/7, 6/8	8/10, S	S, S
3	A	0	7/9, 8/10	S, S	S, S	…	…	…	…	…	…	…
3	A	24	…	…	…	…	…	6/8, 6/8	S, S	S, S	…	…
3	B	0	…	…	…	S, S	S, S	…	…	…	…	…
3	B	24	…	…	…	…	…	6/7, 7/9	…	7/8, 8/10	S, S	S, S

Note.— 7/9* = Guinea-pig paralysed on the seventh day and died on the ninth day.
1/1 m.† = 1 in a million.
S = Guinea-pig remained well.
… = Dilution not tested.
The above abbreviations have been used in all the tables in this paper.

glycine, 2 per-cent sheep-serum and 0·15 per cent peptone. The normal sheep-brain used for the preparation of brain extract and the fixed-virus sheep-brain used as the source of virus inoculum were emulsified in a mortar with sterile glass-powder. Experiments were carried out to determine whether by using a Waring blendor for the emulsification of brain instead of grinding it in a mortar, the concentration of virus obtained in cultures could be increased.

Each of the flasks A and B contained 36 c.c. of 10 per cent steamed sheep-brain extract, 25 c.c. of 20 per cent glycine, 20 c.c. of 1·5 per cent peptone, 4 c.c. of sheep-serum and 101 c.c. of sterile distilled water. Thirty-six c.c. of the supernatant from a centrifugalized 20 per cent suspension of fixed-virus sheep-brain were added to each flask. The brain extract and the virus inoculum used in flask A were prepared by grinding in a mortar with sterile glass-powder, while that in flask B were prepared with a blendor. The cultures were incubated under strict anaerobic conditions in a McKintosh and Fildes's anaerobic jar for 24 hours at 37°C. The virus content of the cultures before and after incubation was titrated in guinea-pigs. The results of three such experiments are given in Table I.

It will be seen from Table I that when the inoculum was prepared by grinding the infected brain in a mortar, the initial concentration of the virus in cultures before incubation varied from 10,000 to 15,000 m.l.d. per c.c. When the inoculum was prepared with a Waring blendor, it was possible to obtain a concentration of 50,000 m.l.d. per c.c. under the same conditions of test. After 24 hours' incubation the virus content of cultures grown in a medium containing brain extract and virus inoculum prepared by grinding was 2·5 million m.l.d. per c.c. When brain extract and virus inoculum were prepared with a blendor, concentrations of virus varying from 5 to 10 million m.l.d. per c.c. were obtained.

The advantage of using a Waring blendor for obtaining higher concentrations of virus in cultures having been established, this method of emulsification of brain substance, for preparation of sheep-brain extract and virus inoculum, was adopted in all subsequent work.

Effect of adding tryptophan.

Experiments were carried out to determine whether the concentration of rabies virus obtained in cultures could be increased by the addition of tryptophan to the medium.

Flasks A, B and C contained the following :—

	A c.c.	B c.c.	C c.c.
Steamed sheep-brain extract, 10 per cent ...	30·0	30·0	30·0
Glycine, 20 per cent	25·0	25·0	25·0
Peptone, 1·5 per cent	20·0	20·0	20·0
Sheep-serum	4·0	4·0	4·0
Tryptophan	...	2·0	20·0
Distilled water	101·0	99·0	81·0

Each flask was inoculated with 36 c.c. of the supernatant from a centrifugalized 20 per cent suspension of fixed-virus sheep-brain. The virus content of the cultures before and after incubation anærobically was titrated in guinea-pigs. The results are given in Table II :—

TABLE II.

Flask.	Number of hours at 37°C.	Fate of guinea-pigs inoculated subdurally with 0·2 c.c. of culture in dilutions.					
		1/10.000	1/20,000	1/1 m.	1/2 m.	1/5 m.	1/10 m.
A	0	7/9, 9/10	S, S	...	...	...	...
	24	...	...	7/8, 10/12	8/10, S	S, S	...
B	24	...	...	7/8, 7 8	6/7, 6/8	8/9, S	S, S
C	24	...	...	6/7, 7,9	5,7, 5,7	6/8, 6/8	S, S

For explanation of abbreviations used, *ece* 'note' under Table I.

It will be seen from Table II that the initial virus content of the cultures as determined by the virus content of flask A before incubation was 50,000 m.l.d. per c.c. The virus content of culture A at the end of 24 hours' incubation was 10 million m.l.d. per c.c., while that of flasks B and C was 25 million m.l.d. per c.c. These results would appear to indicate that (*i*) tryptophan has a stimulating effect on the growth of rabies virus in cultures and (*ii*) this growth-promoting effect is the same whether tryptophan is added in a concentration of 2 mg. or 20 mg. per 100 c.c. of the medium. A concentration of 2 mg. per cent of tryptophan was added to the culture medium in all subsequent work.

Effect of adding certain accessory factors.

The observation that tryptophan increased the concentration of virus in cultures suggested that the addition of accessory factors might prove beneficial for the multiplication of the virus. In the first experiment the effect of each of the factors when added to the medium in a concentration of 2 μg. per c.c. was investigated. Owing to the difficulty of obtaining adequate supplies of animals no attempt was made to determine the optimum concentration of each of the factors required.

Flasks A, B, C, D, E and F contained the following :—

	A c.c.	B c.c.	C c.c.	D c.c.	E c.c.	F c.c.
Steamed sheep-brain extract, 10 per cent ...	7·50	7·50	7·50	7·50	7·50	7·50
Glycine, 20 per cent	6·25	6·25	6·25	6·25	6·25	6·25
Peptone, 1·5 per cent	5·00	5·00	5·00	5·00	5·00	5·00
Sheep-serum	1·00	1·00	1·00	1·00	1·00	1·00
Tryptophan	0·50	...	...	...	...	...
Thiamine hydrochloride	...	0·10	...	...	...	...
Nicotinic acid	...	...	0·10	...	...	...
Pyridoxine hydrochloride	...	...	...	0·10	...	...
Calcium pantothenate	...	...	...	...	0·10	...
Riboflavin	...	...	...	...	...	0·10
Distilled water	24·75	25·15	25·15	25·15	25·15	25·15

Nine c.c. of the supernatant from a centrifugalized 20 per cent suspension of fixed-virus sheep-brain were added to each flask. The flasks were incubated anærobically for 48 hours at 37°C. The virus content of the cultures before and after incubation is given in Table III :—

<div align="center">TABLE III.</div>

Flask.	Number of hours at 37°C.	Fate of guinea-pigs inoculated subdurally with 0·2 c.c. of culture in dilutions.					
		1/10,000	1/20,000	1/5 m.	1/10 m.	1/15 m.	1/20 m.
A {	0	7/9, S	S, S	...	...	...	...
	48	...	...	9/10, S	S, S	...	...
B	48	...	...	8/10, S	5/7, 7/8	7/9, S	S, S
C	48	...	...	6/8, 7/8	5/7, S	S, S	S, S
D	48	...	...	5/7, 7/8	6/8, 7/9	6/8, 7/9	S, S
E	48	...	...	6/8, 7/9	6/8, 8/10	6/8, S	S, S
F	48	...	...	7/9, 8/10	8/10, S	S, S	S, S

For explanation of abbreviations used, *see* 'note' under Table I.

It will be seen from Table III that the initial virus content of the cultures was 50,000 m.l.d. per c.c. The highest concentration of virus obtained with tryptophan was 25 million, with thiamine hydrochloride 75 million, with nicotinic acid 50 million, with pyridoxine hydrochloride 75 million, with calcium pantothenate 75 million and with riboflavin 50 million m.l.d. per c.c. These results showed that each of the factors in the concentration tested had growth-promoting properties, the most effective ones being thiamine hydrochloride. pyridoxine hydrochloride and calcium pantothenate.

A second experiment was carried out to determine whether higher concentrations of the virus could be obtained in cultures by the addition of all the above factors together.

Flasks A and B contained the following :—

	A c.c.	B c.c.
Steamed sheep-brain extract, 10 per cent ...	30·0	30·0
Glycine, 20 per cent	25·0	25·0
Peptone, 1·5 per cent	20·0	20·0
Sheep-serum	4·0	4·0
Tryptophan	2·0	2·0
Thiamine hydrochloride	...	0·4
Nicotinic acid	. .	0·4
Pyridoxine hydrochloride	.	0·4
Calcium pantothenate	...	0·4
Riboflavin	...	0·4
Distilled water	99·0	97·0

The accessory factors were added just before adding the virus inoculum. The cultures were inoculated with 30 c.c. of the supernatant from a centrifugalized 20 per cent suspension of fixed-virus. The initial virus content of the cultures was determined and the flasks incubated anærobically at

37°C. The virus content of the cultures after 24 hours' incubation was titrated in guinea-pigs. The results are given in Table IV :—

TABLE IV.

Flask.	Number of hours at 37°C.	Fate of guinea-pigs inoculated subdurally with 0·2 c.c. of culture in dilutions.							
		1/10,000	1/20,000	1/2 m.	1/5 m.	1/10 m.	1/20 m.	1/50 m.	1/100 m.
A	0	7/9, S	S, S	...	...	...	...	...	...
	24	...	...	6/8, 8/10	5/7, S	S, S	...	...	...
B	24	...	...	...	...	...	5/7, 6/8	6/8, S	S, S

For explanation of abbreviations used, *see* 'note' under Table I.

It will be seen from Table IV that the initial virus content of the cultures was 50,000 m.l.d. per c.c. The virus content of culture A, grown in a medium devoid of the accessory factors, was 25 million m.l.d. per c.c. at the end of 24 hours' incubation, while that of flask B containing all the five accessory factors was 250 million m.l.d. per c.c. These findings would appear to show that as a result of the addition of the accessory factors to the medium it is possible to step up the concentration of virus in cultures to 250 million m.l.d. per c.c.

The effect of adding accessory factors before and after adding the virus inoculum to the medium was also investigated. It was found that the addition of accessory factors to the medium on the day previous to the inoculation of the virus did not yield satisfactory results. The best results were obtained when the factors were added just before or immediately after adding the virus inoculum to the medium.

Effect of not adding normal sheep-brain extract to the medium.

In all the previous experiments normal sheep-brain extract has been used as one of the ingredients of the culture medium. The finding that certain accessory factors had marked growth-stimulating properties on cultures of rabies virus suggested that possibly some of the factors tested might have been supplied by the normal sheep-brain extract used in the medium. An investigation was carried out to determine the effect of eliminating sheep-brain extract from the medium.

It might also be possible that some of the salts present in the steamed sheep-brain extract promoted the growth of virus. Parallel experiments were, therefore, run substituting Tyrode solution for distilled water used in the medium.

Two such experiments were carried out.

Flasks A, B and C contained the following :—

	A c.c.	B c.c.	C c.c.
Steamed sheep-brain extract, 10 per cent ...	30·0	...	...
Glycine, 20 per cent	25·0	25·0	25·0
Peptone, 1·5 per cent	20·0	20·0	20·0
Sheep-serum	4·0	4·0	4·0
Tryptophan	2·0	2·0	2·0
Thiamine hydrochloride	0·4	0·4	0·4
Nicotinic acid	0·4	0·4	0·4
Pyridoxine hydrochloride	0·4	0·4	0·4
Calcium pantothenate	0·4	0·4	0·4
Riboflavin	0·4	0·4	0·4
Distilled water	97·0	127·0	...
Tyrode solution (pH 7·6)	...	. .	127·0

The accessory factors were added just before adding the virus inoculum. Thirty-six c.c. of the supernatant from a centrifugalized 20 per cent suspension of fixed-virus sheep-brain were added to each flask. The pH of the cultures A, B and C was 7·0, 6·8 and 7·4 respectively. It was noticed that when Tyrode solution was added to the culture medium there was a tendency for a precipitate to settle down on standing. When distilled water was used in place of Tyrode solution the cultures were homogeneous with little or no tendency for precipitation. The cultures were incubated

anærobically for 24 hours at 37°C. The virus content of the cultures before and after incubation were titrated in guinea-pigs. The results are given in Table V :—

TABLE V.

Experiment No.	Flask.	Number of hours at 37°C.	Fate of guinea-pigs inoculated subdurally with 0·2 c.c. of culture in dilutions.					
			1/10,000	1/20,000	1/10 m.	1/20 m.	1/50 m.	1/100 m.
1	A	0	8/10, S	S, S	...	...	...	...
	A	24	...	...	...	5/7, 6/8	6/8, S	S, S
	B	24	...	...	...	5/7, 5/7	6/8, 9/10	8/10, S
	C	24	...	...	5/7, 6/8	6/8, 6/8	S, S	S, S
2	A	0	6/8, 7/9	S, S	...	...	...	...
	A	24	...	...	...	6/8, 7/8	6/8, 9/11	S, S
	B	24	...	...	...	7/8, 8/10	6/8, 7/9	6/8, 8/10
	C	24	...	...	...	6/8, 8/10	S, S	S, S

For explanation of abbreviations used, *see* 'note' under Table I.

It will be seen from Table V that the initial virus content of the cultures was 50,000 m.l.d. per c.c. After incubation for 24 hours the virus content of culture A with steamed sheep-brain extract was 250 million m.l.d. and that of culture B without steamed sheep-brain extract 500 million m.l.d. per c.c. The virus content of culture C without steamed sheep-brain extract but containing Tyrode solution in place of distilled water was only 100 million m.l.d. per c.c. The results indicate that when all the accessory factors are added, the use of steamed sheep-brain extract in the medium can be dispensed with and that there is no advantage in substituting Tyrode solution for distilled water in the medium.

Effect of adding biotin.

The effect of the addition of a sample of biotin concentrate prepared in the laboratory on cultures of rabies virus was investigated. The biotin content of the preparation was not known. The concentrate was diluted 1 in 10 with sterile distilled water, sterilized by steaming for half an hour, and 0·2 c.c. of the solution added to every 100 c.c. of the culture.

Flasks A, B, C and D contained the following :—

	A c.c.	B c.c.	C c.c.	D c.c.
Glycine, 20 per cent ...	12·5	12·5	12·5	12·5
Peptone, 1·5 per cent ...	10·0	10·0	10·0	10·0
Sheep-serum	2·0	2·0	2·0	2·0
Tryptophan	1·0	1·0	1·0	1·0
Thiamine hydrochloride ...	0·2	...	0·2	0·2
Nicotinic acid	0·2	...	0·2	0·2
Pyridoxine hydrochloride ...	0·2	...	0·2	0·2
Calcium pantothenate ...	0·2	...	0·2	0·2
Riboflavin	0·2	...	0·2	0·2
Biotin	...	0·2	0·2	0·2
Tyrode solution	63·5	64·3	63·3	...
Distilled water	...	...	...	63·3

The factors were added just before the virus inoculum. Each of the cultures was inoculated with 18 c.c. of the supernatant from a centrifugalized 20 per cent suspension of fixed-virus sheep-brain. The cultures were incubated anaerobically for 24 hours at 37°C. The virus content of the cultures before and after incubation was titrated in guinea-pigs. The results are given in Table VI :—

TABLE VI.

Flask.	Number of hours at 37°C.	Fate of guinea-pigs inoculated subdurally with 0·2 c.c. of culture in dilutions.							
		1/10,000	1/20,000	1/10 m.	1/15 m.	1/20 m.	1/50 m.	1/100 m.	1/200 m.
A	0	8/10, 13/15	S, S	...	...	...	...	...	...
A	24	...	...	...	...	8/10, 8/10	S, S	S, S	S, S
B	24	...	...	7/9, 8/10	6/8, 7/9	6/8, S	S, S	S, S	...
C	24	...	...	...	...	6/8, 7/8	9/10, S	7/9, 8/10	S, S
D	24	...	...	...	...	6/8, 6/8	6/8, 6/8	8/9, S	6/8, 8/10

For explanation of abbreviations used, *see* 'note' under Table I.

These experiments show that under the above conditions concentrations of 2,500 to 5,000 million m.l.d. per c.c. of rabies virus can be easily obtained in cultures.

Effect of incubating cultures under aerobic and strict anaerobic conditions.

Experiments were carried out to determine the effect of incubating cultures grown in the above medium under aerobic and anaerobic conditions.

Each of the flasks A and B contained 25 c.c. of 20 per cent glycine, 20 c.c. of 1·5 per cent peptone, 4 c.c. of sheep-serum, 2 c.c. of tryptophan solution and 126·6 c.c. of distilled water. Thirty-six c.c. of the supernatant from a centrifugalized 20 per cent suspension of fixed-virus sheep-brain were added to each flask. Immediately after the addition of virus inoculum all the accessory factors were added to the cultures in 0·4 c.c. amounts. Flask A was incubated aerobically and flask B under strict anaerobic conditions. The virus content of the cultures before and after 24 and 48 hours' incubation was titrated in guinea-pigs. The results are given in Table VII :—

TABLE VII.

Flask.	Mode of incubation.	Number of hours at 37°C.	Fate of guinea-pigs inoculated subdurally with 0·2 c.c. of culture in dilutions.						
			1/10,000	1/20,000	1/50 m.	1/100 m.	1/200 m.	1/400 m.	1/500 m.
A	Ærobic	0	8/10, S	S, S	...	...	...	...	...
A	,,	24	...	..	6/8, S	6/7, 6,8	6/8, 7/8	8/9, S	S, S
A	,,	48	...	...	8/10, S	6/7, 8/9	6/7, S	S, S	S, S
B	Anærobic	24	...	...	...	5 7, 6/8	6/7, 6/8	8/9, 8/9	6/7, 8/9
B	,,	48	...	...	...	6/8, 7,9	7/9, S	S, S	S, S

For explanation of abbreviations used, *see* ' note ' under Table I.

It will be seen from Table VII that the initial virus content of the cultures was 50,000 m.l.d. per c.c. The virus content of culture A incubated under aerobic conditions for 24 and 48 hours was 2,000 million and 1,000 million m.l.d. per c.c. respectively, while that of culture B after 24 and 48 hours' incubation anaerobically was 2,500 million and 1,000 million m.l.d. per c.c.

The above experiment was repeated. In this experiment the virus content of the culture incubated aerobically was 1,000 million m.l.d. per c.c. after 24 hours and less than 1,000 million m.l.d. per c.c. after 48 hours' incubation. Under strict anaerobic conditions the virus content of the culture after 24 and 48 hours' incubation was 5,000 million and 1,000 million m.l.d. per c.c. respectively.

These results indicate that although high concentrations of virus can be obtained when cultures are incubated aerobically, the use of strict anaerobic conditions is more favourable for the multiplication of the virus.

Cultivation of rabies virus in a cell-free medium.

Evidence was presented by the author (Veeraraghavan, 1946a) to show that rabies virus can multiply in a cell-free medium containing steamed sheep-brain extract, glycine, peptone and filtered serum. The virus inoculum used in the experiments was filtered through a Sterimat F.C.B. grade pad to eliminate the possibility of its containing any intact cells which might be responsible for the multiplication of the virus.

Preliminary experiments showed that there was a considerable fall in the virus content of the supernatant from a centrifugalized 20 per cent suspension of fixed-virus sheep-brain after filtration through a Sterimat F.C.B. grade pad. During the course of the present investigation it was found that in order to obtain high concentrations of the virus in cultures the virus content of the inoculum should be heavy. Therefore, in the present series of experiments using filtered virus inoculum, one part of the filtrate was added to one part of the medium, instead of the usual proportion of one part of the virus to five parts of the medium. The final concentration of the various constituents in the medium was, however, adjusted to be the same.

A culture medium containing 5 per cent glycine, 0·3 per cent peptone and 4 per cent sheep-serum in distilled water was prepared. A 20 per cent emulsion of fixed-virus sheep-brain was prepared in a Waring blendor and centrifuged for one hour at 3,000 r.p.m. The top layer of the supernatant was filtered through a Sterimat F.C.B. grade pad. Twenty-five c.c. of the filtrate were added to 25 c.c. of the culture medium so that the final concentration of the various constituents was the same as that generally used. To this was added 0·5 c.c. of tryptophan solution and all the accessory factors including biotin in 0·1 c.c. amounts. The virus content of the culture before and after incubation anaerobically for 24 hours was titrated in guinea-pigs.

The culture before incubation was infective in a dilution of 1 in 20,000 indicating that its virus content was 100,000 m.l.d. per c.c. After incubation for 24 hours the highest infective dilution of the culture was 1 in 500 million indicating that its virus content was 2,500 million m.l.d. per c.c.

The above experiment was repeated with identical results.

These findings would appear to show that rabies virus can pass through a Sterimat F.C.B. grade pad and is able to multiply in a cell-free medium.

Effect of filtering virus inoculum through Sterimat F.C.B. and G.S. grade pads.

The supernatant from a centrifugalized 20 per cent suspension of fixed-virus sheep-brain was first filtered through a Sterimat F.C.B. grade pad and then through a Sterimat G.S. pad. The filtrate so obtained was water-clear and was used as the virus inoculum and the experiment repeated.

In this experiment the culture before incubation was not infective in a dilution of 1 in 10 and the 24 hours' culture was not infective in a dilution of 1 in 1,000, the lowest dilution tested. These results were probably due to the fact that there was practically little or no virus in the inoculum used for seeding the medium.

Serial passage of virus in vitro.

Several attempts were made but without success to passage the virus in a culture medium containing 2·5 per cent glycine, 0·15 per cent peptone, 2 per cent

sheep-serum, 2 mg. per cent of tryptophan, 2 μg. per c.c. of each of thiamine hydrochloride, nicotinic acid, pyridoxine hydrochloride, calcium pantothenate and riboflavin and 0·2 c.c. per 100 c.c. of biotin solution. Although high concentrations could be obtained in the first 24 hours' culture, this was not possible in the subsequent sub-passages *in vitro*, the reason possibly being that the initial virus inoculum used in the first culture contained some factor or factors essential for the growth of the virus. Experiments were, therefore, carried out to determine whether the addition of an equal amount of normal sheep-brain, either steamed or filtered through a Sterimat F.C.B. grade pad, to the second, third and fourth cultures would help in the multiplication of the virus during serial passage.

The supernatant from a centrifugalized 20 per cent suspension of fixed-virus sheep-brain was filtered through a Sterimat F.C.B. grade pad. Twenty-five c.c. of the filtrate were inoculated into 25 c.c. of a medium containing 5 per cent glycine, 0·3 per cent peptone and 4 per cent sheep-serum in distilled water. To this was added 0·5 c.c. of tryptophan solution and all the accessory factors in 0·1 c.c. amounts. The culture was incubated anærobically for 24 hours at 37°C. The virus content of the culture before and after incubation was titrated in guinea-pigs.

The second passage was carried out as follows : Nine c.c. of the 24 hours' culture were added to a medium containing 9 c.c. of 20 per cent steamed sheep-brain extract, 6·25 c.c. of 20 per cent glycine, 5 c.c. of 1·5 per cent peptone, 1 c.c. of sheep-serum, 0·5 c.c. of tryptophan solution and 22·75 c.c. of distilled water. All the accessory factors were added in 0·1 c.c. amounts and the culture incubated for 24 hours anærobically. Subsequent passages were carried out by adding 9 c.c. of the culture to another flask containing 45 c.c. of the medium described above. The fourth passage culture after 24 hours' incubation was titrated in guinea-pigs.

A parallel experiment was run using the filtrate of a 20 per cent suspension of the same normal sheep-brain in the medium instead of steamed sheep-brain extract.

The initial virus content of the first culture was 100.000 m.l.d. per c.c. After 24 hours' incubation it was 2.500 million m.l.d. per c.c. After four serial passages the virus content of the culture using a medium containing steamed sheep-brain extract was 500 million m.l.d. per c.c.. while that with filtered sheep-brain extract was 50 million m.l.d. per c.c.

These results indicate that it is possible to propagate the virus in cultures *in vitro*.

Immunizing value of culture virus.

Veeraraghavan (1946*b*) has shown that culture vaccine whose virus content is adjusted to be equal to that of 5 per cent Semple's vaccine affords a high degree of protection against fixed-virus given subdurally or street virus given intramuscularly.

Immunity experiments have now been carried out with vaccines prepared from cultures containing much higher concentrations of rabies virus than those reported previously. The results obtained confirm the previous finding that it is possible to immunize experimental animals with culture-virus vaccine against a subsequent infection with street virus. Encouraging results have also been obtained in post-infection treatment of animals given street virus into neck muscles when a phenol-killed culture vaccine of high virus content is used for treatment. Details of these investigations, now in progress, will form the subject of a separate communication.

DISCUSSION.

It will be seen from the observations recorded in this paper that very high concentrations of rabies virus can be obtained in cultures *in vitro* in a medium containing 2·5 per cent glycine, 0·15 per cent peptone, 2 per cent sheep-serum, 2 mg. per cent of tryptophan and 2 μg. per c.c. of each of the accessory factors thiamine hydrochloride, nicotinic acid, pyridoxine hydrochloride, calcium pantothenate and riboflavin and 0·2 c.c. per 100 c.c. of biotin solution. The initial concentration of the virus in cultures with the above medium is generally in the neighbourhood of 50,000 m.l.d. per c.c. After incubation anærobically for 24 hours at 37°C. the titre of the culture rises to 5,000 million m.l.d. per c.c., a 100,000-fold increase in the titre of the virus. With the addition of all the accessory factors it is possible to dispense with the addition of steamed sheep-brain extract to the medium.

The highest initial virus content of the cultures, which represents a 1 in 30 dilution of the infected sheep-brain, was 50,000 m.l.d. per c.c. Therefore, the highest concentration of virus generally obtained in the brains of infected sheep used in the manufacture of antirabic vaccine will be 50,000×30, i.e. 1,500,000 m.l.d. per c.c. Using the method of cultivation now described it is possible to obtain concentrations of 5,000 million m.l.d. per c.c. representing a 3,300-fold increase in the concentration of virus ordinarily obtained in the brains of infected sheep.

The concentration of virus obtained in cultures incubated under strict anærobic conditions was higher than that incubated under ærobic conditions.

A number of workers have shown that rabies virus can be cultivated in a medium containing rabbit, mouse, chick or rat embryo brain tissue with serum-Tyrode solution (Kanajawa, 1936, 1937; Webster and Clow, 1936, 1937; Bernkopf and Kligler, 1937; Raymond Parker and Hollender, 1945). Experimental evidence has been presented in this paper to show that it is possible to cultivate rabies virus in a medium devoid of living cells. The concentration of virus obtained in cultures, assessed in terms of minimum lethal doses for the guinea-pig, is much higher than that reported by others using mice as experimental animals. The successful cultivation and propagation of the ætiological agent of rabies in a cell-free medium supports the view put forward by Veeraraghavan (1944, 1945) that an agent other than a virus may be connected with the ætiology of the disease.

Webster (1939) found that commercial vaccines inactivated with phenol or chloroform afford protection to mice if given intraperitoneally and in quantities approximating five times the dose advocated per gramme of body-weight in man. Veeraraghavan (1946b) has shown that when five times the comparable dose per gramme of body-weight of phenol vaccine or culture vaccine with the same virus content is given intraperitoneally to rabbits or guinea-pigs a considerable degree of immunity is produced. All the available evidence indicates that antirabic vaccine ' is effective only provided it is given repeatedly and in large doses, totaling at least 1 per cent of the body-weight ' (Wilson and Miles, 1946). On this basis the total quantity of vaccine necessary for producing adequate immunity in man would be 700 c.c. the administration of which is out of question. Even if it were possible to give such a large quantity of vaccine, the amount of nerve tissue protein administered would be increased very considerably with the associated risk of the patient developing post-treatment paralysis. With the culture vaccine,

however, it should be possible to administer in a fraction of a c.c. the virus content of 700 c.c. of 5 per cent Semple's vaccine. The culture vaccine has the added advantage that its virus content can be accurately determined and is free from gross nerve tissue. The nerve-tissue protein administered is reduced to a negligible fraction as the protein nitrogen content of 1 c.c. of culture vaccine is less than that of 1 c.c. of 5 per cent Semple's vaccine.

SUMMARY.

1. Tryptophan, thiamine hydrochloride, nicotinic acid, pyridoxine hydrochloride, calcium pantothenate, riboflavin and biotin concentrate have marked growth-stimulating properties on cultures of rabies virus *in vitro.*

2. It is possible to obtain concentrations of 5,000 million m.l.d. per c.c. of rabies virus in cultures *in vitro* in a medium containing 2·5 per cent glycine, 0·15 per cent peptone, 2 per cent sheep-serum. 2 mg. per cent tryptophan. 2 μg. per c.c. of each of thiamine hydrochloride, nicotinic acid, pyridoxine hydrochloride. calcium pantothenate and riboflavin with biotin.

3. The concentration of virus obtained in cultures is nearly 3,300 times that ordinarily obtained in the brains of infected sheep used in the manufacture of antirabic vaccine.

4. The successful cultivation of the ætiological agent of rabies in a cell-free medium suggests that it is not a virus in the accepted sense of the term.

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A SHORT NOTE ON A SALMONELLA POSSESSING SOMATIC ANTIGENS III, X, XXVI, FLAGELLA ANTIGENS PHASE I UNDETERMINED AND PHASE II, 1, 5.

BY

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THE study of Salmonellas is one of the most interesting branches in bacteriology. It is regrettable that a Salmonella Centre such as the Salmonella Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N. W. 9, does not exist in this sub-continent. The International Salmonella Centre at Copenhagen, where a great deal of work on Salmonellas has been done, is said to have several subsidiary laboratories under its control. It is now felt that this important branch in bacteriology should find a place in one of the research institutes in India where confirmation or identification of Salmonellas isolated by any one either from man, birds or animals could be carried out together with the epidemiology of the Salmonella group of infections.

There must be many instances where an organism isolated has not been identified or incorrectly identified. If a Salmonella Centre could be brought into existence these anomalies would not occur.

Confirmation and identification of Salmonellas are amongst the many functions of the Central Military Pathological Laboratory, Poona. Most of the Salmonella cultures are received from military laboratories dependent on this institution for their biological products, while a very small percentage of cultures are received from civil laboratories.

A culture was received from a civil laboratory for identification. Apart from the fact that the organism was derived from the fæces of a patient in one of the local hospitals, no further particulars are known. In this paper the organism will be referred to as X.

Morphology.—

Gram-negative motile bacillus.

Biochemistry.—

Lactose	...	No change.	Rhamnose ...	Acid and gas.
Glucose	...	Acid and gas.	Inosite ...	,,
Mannite	...	,,	Mannose ...	,,
Saccharose	...	No change.	H_2S ...	+
Dulcite	...	Acid and gas.	Indol ...	Negative.
Arabinose	...	,,		

The fermentation of the above carbohydrates was noted after 24 hours' incubation. As will be seen from the above findings, the biochemical reactions of the organism are those of a Salmonella.

Serology.—

Identification of the somatic antigen:

Slide agglutination: Using an alcoholized suspension of the organism the following are the results of slide agglutination against the undermentioned sera :—

Group A ... *S. paratyphosum* A ... ... No agglutination.
I, II, XII

,, B ... *S. stanley* ... ... ,,
IV, V

,, C_1 ... *S. paratyphosum* C .... ,,
VI, VII

,, C_2 ... *S. newport* ... ... ,,
VI, VIII

,, D ... *S. enteritidis* ... ... ,,
I, IX, XII

,, E_1 ... *S. anatum* ... ... Agglutination.
III, X, XXVI

,, E_2 ... *S. newington* ... ... ,,
III, XV

,, E_3 ... *S. senftenberg* ... ... ,,
I, III, XIX

,, F ... *S. poona* ... ... No agglutination.
XIII, XXII

Dreyer's agglutination test.—

Alcoholized suspension of :—

Serum.	*S. anatum.*	*S. newington.*	*S. senftenberg.*	X.
S. anatum ...	2,000	1,000	250	2,000
S. newington ...	256	2,048	128	256
S. senftenberg ...	1,000	1,000	1,000	1,000

It will be seen that due to the presence of the somatic antigen III present in all the members of the E group, there is a great deal of cross agglutination between groups E_1 represented by *S. anatum*, E_2 represented by *S. newington* and E_3 by *S. senftenberg.*

An attempt was, therefore, made to prepare a monospecific serum by eliminating the common somatic agglutinin III. The following results were obtained :—

Alcoholized suspension of :—

Serum.	S. anatum.	S. newington.	S. senftenberg.	X.
S. anatum X, XXVI ...	+	0	0	+
S. newington XV ...	0	+	0	0
S. senftenberg ...	0	0	+	0

The above table is self-explanatory in that the monospecific *S. anatum* serum agglutinates its homologous antigen in addition to the somatic antigens contained in X.

S. newington and *S. senftenberg* somatic antigens are agglutinated by their respective agglutinins alone.

It is presumed that the somatic antigen contained in X organism is similar to that in *S. anatum.*

Identification of the heat-labile antigen : The organism was plated and six colonies seeded into broth—two such colonies of the cultures were agglutinated by *S. cholerae suis* var. *kunzendorf* serum, while the other four were not agglutinated. A concentrated suspension from each type of the flagellar phases was made and standardized to 6,400 million organisms per c.c.

Phase 1.—

This phase of the organism was not agglutinated by ' II ' sera of the following organisms :—

S. paratyphosum A ...	...	...	Flagellar agglutinin	" a "
S. paratyphosum C ...	...	,,	,,	Phase I " c "
S. typhosum ...	...	,,	,,	" d "
S. newport ...	...	,,	,,	Phase I " e, h "
S. enteritidis ...	...	,,	,,	" g, m "
S. typhi-murium ...	...	,,	,,	Phase I " i "
S. thompson ...	...	,,	,,	Phase I " k "
S. litchfield ...	...	,,	,,	Phase I " l, v "
S. virchow ...	...	,,	,,	Phase I " r "
S. bareilly ...	...	,,	,,	Phase I " y "

It was further not agglutinated by polyvalent sera containing phase I and II agglutinins of the undermentioned organisms :—

S. typhi-murium, S. newport, S. thompson, S. morbificans bovis, S. london, S. bareilly, S. lexington, S. kentucky, S. cerro, S. ballerup, S. abortus equi.

The only flagellar agglutination that could be obtained with the phase I suspension of the organism was with *S. paratyphosum* B 'H'. The following are the results using this serum :—

S. paratyphosum B 'H' phase I *versus* *S. paratyphosum* B 'H'   = 5,000
S. paratyphosum   ,,   ,,   :,   X 'H' phase I   = 500
S. paratyphosum   ,,   ,,   ,,   *S. schleissheim* "b, Z_{12}" = 500

Only 1/10 of the flagellar antigen phase I present in the organism is similar to that of *S. paratyphosum* B 'H' phase I.

A suspension of phase I antigen of the organism was now injected into a rabbit. The following are the titres obtained :—

X 'H'   *versus*   X 'H' phase I   ...   ... = 5,000
X 'H'   ,,   X 'O'   ...   ... = 1,000
X 'H'   ,,   X 'H' phase II   ...   ... = 125
X 'H'   ,,   *S. para* B 'H' phase I   ... = 500
X 'H'   ,,   *S. schleissheim* "b, Z_{12}"   ... = 500
X 'H'   ,,   *S. cholerae suis* 'H' var. *kunzendorf*   ...   . = 25
X 'H'   ,,   *S. typhi-murium* var. *binns* ... = 0

A 'H' suspension of the following organisms was not agglutinated by X 'H' serum :—

S. paratyphosum A, *S. paratyphosum* C phase I, *S. typhosum, S. newport* phase I, *S. enteritidis, S. typhi-murium* phase I, *S. thompson* phase I, *S. litchfield* phase I, *S. virchow* phase I, *S. bareilly* phase I, *S. poona* phase I, *S. cerro, S. dusseldorf, S. simsbury, S. uganda* phase I, *S. lexington* phase I, *S. ballerup, S. tennessee, S. abortus equi.*

It is seen from the mirror agglutination that the heat-labile agglutinin "b" contained in X 'H' serum is responsible for 1/10 of the titre of the serum, the remaining 9/10 has still to be determined.

Below are the agglutination titres of the somatic agglutinins present in X 'H' serum :—

Alcoholized suspension of :—

Serum.	S. anatum.	S. newington.	S. senftenberg.	X.
X	1,000	500	128	1,000

When X 'H' serum is deprived of its somatic agglutinin III, this monospecific serum agglutinates not only its homologous antigen but also the somatic antigens

contained in *S. anatum.* The antigens contained in *S. newington* and *S. senftenberg*, however, remain unaffected.

It is, therefore, conclusively proved that the somatic antigen contained by the organism under investigation contains the factors III, X, XXVI.

Identification of phase II heat-labile antigen :

As previously mentioned a concentrated suspension to contain 6,400 million organisms per c.mm. was made.

The following are the agglutination titres obtained against the undermentioned sera :—

S. typhi-murium var. *binns*	*versus*	*S. typhi-murium* var. *binns*	...	= 200
..	.,	.,	,,	*S. choleræ suis* var. *kunzendorf*	= 100
.,	,,	,,	.,	X ' H ' phase II ...	...	= 100
S. choleræ suis var. *kunzendorf*		*S. choleræ suis* ' H ' var. *kunzendorf* ...	...	= 250
,.	.,	..	.,	*S. typhi-murium* var. *binns*	...	=<50
,.	.,	..	,.	X ' H ' phase II ...	...	= 250

Absorption test :

1. The agglutinins contained in *S. typhi-murium* var. *binns* serum are not completely absorbed by a suspension of X ' H ' phase II antigen. The homologous titre after absorption of the serum is, however, reduced from a titre of 1 : 250 to one of 1 : 125.

2. When a serum of *S. choleræ suis* ' H ' var. *kunzendorf* is absorbed with a suspension of X ' H ' phase II, there is complete absorption of all the agglutinins.

Monovalent sera containing phase II agglutinins .2, 3 and agglutinin 5 were prepared.

Phase II of X organism was not agglutinated by the serum containing agglutinins 2, 3 but was readily agglutinated by the serum containing agglutinin 5.

A serum was prepared from phase II of X organism. The following are the agglutination titres :—

X ' H ' phase II	*versus*	X ' H ' phase II ...	...	= 250
..	..	..	.,	*S. choleræ suis* ' H ' var. *kunzendorf* ...	...	= 250
..	..	..	..	*S. typhi-murium* var. *binns*	= 50

X ' H ' phase II agglutinins are completely absorbed by a suspension of *S. choleræ suis* var. *kunzendorf.*

The above agglutination absorption tests are conclusive that X ' H ' phase II contains the antigens I. 5.

SUMMARY.

An organism having the biochemical properties of a Salmonella is described.

Serologically it contains the somatic antigens III, X, XXVI.

Flagellar antigen phase I, besides containing the factor " b ", has some other antigen in addition.

It contains the factors 1, 5 flagellar antigens in phase II.

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HYPERTROPHIC GINGIVITIS IN INDIAN CHILDREN AND ADOLESCENTS.

BY

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AND

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INTRODUCTION.

PERIODONTAL disease is the principal and most serious oral menace to general health. The prevalence in India of these pathologic conditions associated with the investing structures of the teeth must constitute a serious threat to the health of the community. Laboratory findings and clinical observations have proved conclusively that infections arising from the teeth and their supporting tissues are capable of producing pathogenic changes in other parts of the body and of becoming a source of general systemic infections. The end result of untreated periodontal disease is loss of the teeth : but even this serious sequela should be considered of only secondary importance compared with the potentialities of the disease as an ætiological or contributing factor in many constitutional disturbances.

From the incipient stages of a simple gingivitis to the terminal phase of a destructive periodontoclasia, often extending over a long period of years, there is continuous migration of bacteria and absorption of toxins into the circulatory system.

It has been estimated (Hopkins, 1934) that in the average adult mouth containing the full quota of teeth and in a moderately advanced stage of periodontal disease there is an area of approximately 2,490 sq. mm. of necrotic tissues capable of absorbing toxins and bacteria contained in the periodontal pockets. The situation of the oral cavity at the opening of the alimentary tract increases the potentialities for harm of sepsis in this region.

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It is recognized that gingivitis and other periodontal conditions are often the oral manifestations of a variety of systemic diseases, and these oral symptoms of systemic disorders must be carefully differentiated from gingivitis and other periodontal lesions due to other causes. Conversely, the importance of oral foci of infection as ætiologic and contributory factors in many systemic disorders has been adequately established. Among these may be included infections and reflex disorders of the eye (Kravitz, 1946 ; Lowell, 1934 ; Winslow, 1938), heart disease (Haden, 1932), kidney disease (Canby, 1939), duodenal ulcer (Winslow, *loc. cit.*), myositis, neuritis and iritis (Albanese, 1934), anæmia (Barrieu, 1939), diseases of the gastro-intestinal, genito-urinary, respiratory and nervous systems (Gobar, 1937), infectious arthritis (Cecil and Angevine, 1939).

In a study of periodontal disease certain types or phases must be clearly differentiated. Any classification of the commonest types must include : (1) Gingival recession due to abrasion ; (2) Atrophy : (*a*) senile, (*b*) pre-senile, (*c*) from disuse, (*d*) from occlusal trauma ; (3) Gingivitis : (*a*) marginal, (*b*) hypertrophic ; (4) Marginal periodontitis (dirt pyorrhœa) ; (5) Parodontosis (often referred to as ' idiopathic pyorrhœa ') ; (6) A syndrome or combination of marginal parodontitis and parodontosis. These types are not always clearly defined or easily differentiated and several may occur together at the same time in the one mouth.

In gingivitis the bone is not involved, at least in the first instance, whereas in marginal parodontitis active lacunar resorption of the alveolar bone takes place. The resorption is of the horizontal type usually beginning on the crests of the interdental bony septa. In parodontosis, which is believed to be systemic in origin, resorption of the alveolar bone takes place by vascular resorption and bone reversion (osteolysis) before the gingivæ are involved. Bone resorption is irregular and in a plane vertical to the alveolar margin. Once pocketing occurs the condition becomes complicated by local factors and the syndrome of parodontosis and marginal parodontitis may be the end result.

It is commonly believed that the ætiology of any type of parodontal disease is always mixed. The disease may have a background of systemic predisposing factors, such as mineral or vitamin deficiencies, metabolic or endocrine disturbances ; and may be activated by local irritants, such as toxins, infection, calcareous deposits, faulty oral hygiene and abnormal occlusal stresses. It is also fairly generally accepted that the underlying ætiology of gingivitis and diffuse alveolar bone atrophy is identical, the latter being a chronic destructive complication of a latent gingivitis, the difference being merely a time factor and the extension of the disease process from the soft tissues to the alveolar bone or vice versa.

Periodontal disease is universally extremely prevalent. Even in Western countries it is probably the most prolific of all diseases. Black (quoted by Masuda, 1946) found in America 13 per cent occurrence in patients 20 to 24 years of age, rising progressively to 88 per cent in patients over 50. Clinical observation over a number of years would appear to indicate a much higher incidence of this disease in India ; and the limited amount of statistical research so far reported emphasizes the fact that periodontal disease is without doubt the commonest of all pathologic conditions in this count·

From available literature it would seem that investigations relative to the incidence of periodontal disease in India have been confined to Northern India. An investigation by Day and Shourie (1944) into the incidence of parodontal disease in children and adults revealed that 75 per cent of children suffered from gingivitis as early as 10 years of age ; by 30 years of age 62 per cent of one of the healthiest groups to be found in the Punjab, viz. police constables, suffered from chronic suppurative periodontitis, and this figure increased to 90 per cent in the 40 to 50 age group.   Day and Tandon (1940) found 69 per cent of a group of 756 Lahore school children affected with gingivitis.   In Hissar (famine area), Kasur (chronic endemic fluorosis area), and Kangra valley (rickets and osteomalacia area), Day found 85, 55 to 85, and 81 per cent respectively in various age groups from 5 to 18 years suffering from gingivitis, mainly of the hypertrophic variety.

In view of the universally high incidence of these disease conditions noted in various parts of the Punjab province it was thought desirable to carry out further investigations using more accurate methods of assessment and directed specifically towards obtaining reliable data on the incidence of periodontal disease in various age groups.   Careful clinical observation over a long period of years along with the fact that the incidence of gingivitis decreases with age while that of chronic suppurative periodontitis increases strongly suggests that the very prevalent hypertrophic gingivitis among children progresses to the chronic suppurative form characterized by pocket formation and diffuse alveolar bone atrophy in late adolescent and adult life.

As a first step in a more comprehensive study of periodontal conditions in India it was decided to undertake an investigation into the incidence of gingivitis and relative factors in a large group of children of school age.

MATERIAL AND METHODS.

It was considered that a reliable index of gingival health and disease in children and young adolescents could be obtained by studying conditions in schools attended by boys and girls of an age ranging from 6 to 20 years.   With the consent and co-operation of the principals, the Islamia High School and Queen Mary College, Lahore, were selected for our purpose.   In the Islamia High School, which is a middle-class boys school, 1,054 subjects from 9 to 17 years were examined ; in Queen Mary College, which is attended mostly by girls from 5 to 24 years and some boys from 5 to 11 years (the sons and daughters of ruling princes and well-to-do families), 323 subjects were examined, the group being made up of 261 girls and 62 boys.

The investigation, therefore, covered a total of 1,377 subjects constituting a reasonably typical cross-section of the Lahore population of this age and adequate in number for a detailed study of the clinical aspects of the problem.

In the few previous surveys of this kind in India to which reference has already been made, the investigations were in most cases directed towards other problems and not specifically towards a study of gingival conditions : nor were very precise methods used for assessing and recording gingival disease.   Otherwise it is believed that the incidence figures reported in the earlier investigations would probably have been even higher.

In order to obtain a satisfactory basis for comparison of gingival status in India and elsewhere the criteria suggested by King (1945) for gingival health and disease were followed as closely as possible. The following criteria for a ' normal ' condition of the gingivæ can be accepted for Indian subjects : a gum margin tapering off to the tooth surface to which it is firmly adherent, the papillæ almost entirely filling the interdental spaces and rising to a point in the region of the contact point between the adjacent teeth ; the enamel-cement junction being not visible and with no detectable pockets ; the surface of the gum of matt texture just distant from the gum margin with the presence of numerous regularly distributed small depressions giving the tissue an appearance somewhat resembling the peel of an orange. In the Indian subject, however, the normal gum is not always ' uniformly pale pink'. It may present a variety of deeper colourings, sometimes, but not always, in conformity with the various shadings of the skin pigmentation. This fact makes it extremely difficult to establish a ' normal ' colour standard for the oral tissues. Furthermore, in a high percentage of cases the gum tissue may present a uniform black or brown pigmentation or patches of this pigmentation sometimes extending downwards as far as the interdental papillæ. These two phenomena render it difficult to assess normality in terms of colour to evaluate the severity of disease from the degree of injection or reddening, and also greatly reduce the diagnostic value of digital pressure recovery tests.

The clinical signs used for the assessment of the presence and severity of gingival disease with the necessary limitations already mentioned were (*a*) Surface : disappearance of matt surface with reduction or absence of stippling effect. (*b*) Gum margins : swelling, colour change, detachment from tooth surface with deepening of the gingival crevice ; pocket formation and, in late stages, suppuration ; sometimes reddening and ulceration. (*c*) Papillæ : swelling and colour changes, which may be confined to the papillæ or associated with similar changes in other parts of the gum margin ; blunting of the normal pointed papillary tip ; detachment from the adjacent teeth with interdental pocketing. Hæmorrhage may occur from any part of the gum margin, including the papillæ, either spontaneously or on pressure.

The signs of gingival disease will vary according to the type of gingivitis studied and in the acute, subacute and chronic phases of the disease. Animal experiments have shown that differences in contour, colour and texture of the marginal gum are dependent upon the degree of sub-surface epithelial proliferation (King, 1944).

The examination procedure entailed a careful examination of the mouth as a whole with mirror and explorer, and the use of a calibrated blunt probe for estimation of pocket depth. The examination was conducted either in good natural light in the open air or by satisfactory artificial illumination.

In order to test the value under Indian conditions of the procedures outlined by King, a small section of the whole group, approximately 10 per cent, were examined by the precise method advocated, concentrating upon the eight gingival regions between the canines and lateral incisors and between the lateral and central incisors of both upper and lower jaws. The Chart shown was used for these precise observations (*see* end of paper).

Data was sought relative to the condition of the gums, presence of dental caries, tartar, food débris, plaques, malocclusion and cleansing habits. The presence and degree of gingival disease were assessed by observation of colour, texture, gum margins and papillæ, exposure of the enamel-cement junction, depth of lateral, lingual and interdental pockets and presence or absence of suppuration. Digital pressure was used to test for colour recovery and hæmorrhage.

Results and discussion.

(i) *Type of gingival disease.*—From general observation and the evidence produced in this survey of 1,377 children and adolescents it can be stated that the type of gingivitis encountered in Northern India is almost entirely of the hypertrophic variety. Hypertrophic gingivitis is characterized by an enlargement of the gingival tissues and may be either proliferative or œdematous in nature. In the proliferative type there is an increase in tissue elements rather than an increase of fluid in the cell interstices. The tissue is firm and fibrous and does not tend to bleed easily. This is the type most commonly met with in India and universally found in the group under discussion. In many cases it was found that the proliferative type was associated with an œdematous papilla which thus became softer due to seepage of fluid, and of a more bluish or maroon colour than the hard fibrous portion of the gum margin. In the more severe cases the condition sometimes became complicated on the extreme edge of the gingival margin by a reddening or ulceration more characteristic of a marginal gingivitis. It is believed that the œdematous papilla and the marginal ulceration is merely a secondary occurrence following the primary proliferation and due to the presence of irritants such as food débris and calculus against the greater retention area created by the proliferated tissue. In some cases the entire gingivæ of both jaws were involved but in the great majority of cases the anterior gingivæ from central incisors to canines showed a greater incidence and degree of disease.

Where gingival disease was present it was not confined in any case to only one or two of the eight anterior regions examined. In the great majority of cases the disease involved uniformly the whole of the gingivæ of the upper and lower anterior teeth but showed in approximately 7 per cent of subjects a detectable variation in severity of the lesions in the four areas studied in the same jaw, and in 36 per cent a difference in degree of involvement between the upper and lower gingivæ. A definite reddening and ulceration at the extreme margin of the gingivæ, more characteristic of marginal gingivitis, was noted in 26·45 per cent of subjects examined, the incidence being in direct relation to the degree of proliferative gingivitis.

(ii) *Incidence of gingival disease.*—From a comparison of statistics in the available literature and from observations in other countries it would seem evident that the incidence of gum disease in India, at least in the lower and middle classes, is extremely high and probably the highest so far reported in a similar age group. It is also believed that the extent or severity of the lesions is much greater on the average than those ordinarily encountered elsewhere. Of particular interest is the almost universal occurrence of the hypertrophic type of gingivitis, the absence of the recessive type and the appearance of the marginal inflammatory type as only a secondary lesion superimposed on the original hypertrophic from.

In the absence of uniformity in the methods employed in the assessment of gingival disease and in view of the differences in the types of disease found by investigators in the groups examined it is difficult to make a satisfactory comparison of the incidence of gingival disease in various countries or even in similar groups in the same country. In America, McCall (1933) and Brucker (1943) reported strikingly different incidence figures for European children of 98 per cent and 8·7 per cent respectively, in contrast to 3 per cent reported for the American Negro (Sterling, 1928). Balendra (1937) states that gingivitis and pyorrhœa occur in only 20 per cent of those examined in Ceylon. The New Zealand Maori children are said to show a gingivitis incidence of 94 per cent (Saunders and Taylor, 1938). Using very severe diagnostic criteria, King (1945) reported incidence figures for gingivitis in Great Britain ranging from 55 to 97 per cent.

Table I shows the incidence of gingival disease in a group of 1,054 boys of the Islamia High School in Lahore (Punjab Province). The age range was from 9 to 17 years. It will be noted that only 6 of the 1,054 subjects examined showed an absence of gingival disease. Four age groups showed an incidence of 100 per cent and in all groups but one the incidence was over 99 per cent. The average disease incidence for all age groups was 99·43 per cent. In view of the severity of the lesions it was found necessary in assessing the extent of gingivitis to classify the degree of involvement as (*i*) slight to moderate disease, (*ii*) severe disease, (*iii*) very severe disease denoted by +, ++, and +++ gingivitis respectively. The table shows that of the total, 73·62 per cent showed either severe or very severe gum disease, 25·81 per cent slight to moderate gum disease and only 0·57 per cent could be classified as normal. The average disease figure was arrived at by adding the sum of the plus signs and dividing by the number in the group. There would seem to be no significant difference in incidence or disease figure in the various age groups.

Table II shows the gingival status of 261 girls examined in Queen Mary College, Lahore. It will be seen that both the incidence and severity of disease are significantly less than in the Islamia High School (Table I). The age range is different in the two groups, but if only those between the ages of 9 and 17 years are considered in each case, a satisfactory comparison can be made. On this basis it was found that the percentage incidence of disease was 99·43 for the 1,054 boys examined and 73·74 for the 179 girls in the same age range. The average disease figures were 1·94 and 1·21 respectively. In addition to the sex variation there was, as has already been pointed out, a socio-economic difference between these two groups.

As in the case of the boys there was no constant correlation between age and disease figure but in the case of the girls there was a definite though somewhat irregular increase in the disease figure from 11 years onwards corresponding with the age of puberty. This may indicate that there is a hormonal factor concerned in the ætiology of gingivitis at this age as in the common ' pregnancy gingivitis '.

A small group of 62 boys from 5 to 11 years attending Queen Mary College and of the same social status as the girls was also examined for purposes of comparison (Table III). On comparing the gingival condition of 58 boys and 106 girls in the same age range (6 to 11 years) it was found that both the incidence and extent of disease were significantly greater in the boys, the incidence and average

TABLE I.

Gingivitis incidence.

(Islamia High School, Lahore—Boys.)

Age in years.	Number of subjects in group.	Number with no gingivitis.	Gingivitis.			Total with gingivitis.	Gingivitis, per cent.			Per cent with gingivitis.	Average disease figure.
			Slight to moderate.	Severe.	Very severe.		Slight to moderate.	Severe.	Very severe.		
9	9	...	2	5	2	9	22·22	55·55	22·22	100	2·00
10	110	...	24	70	16	110	21·82	63·63	14·55	100	1·93
11	155	1	42	80	32	154	27·10	51·61	20·65	99·36	1·92
12	280	2	64	146	68	278	22·86	52·14	24·29	99·29	2·00
13	181	1	51	100	29	180	28·18	55·25	16·02	99·45	1·87
14	170	1	46	94	29	169	27·06	55·29	17·06	99·41	1·89
15	83	...	20	41	22	83	24·10	49·40	26·51	100	2·02
16	47	...	15	20	12	47	31·91	42·56	25·53	100	1·94
17	19	1	8	10	...	18	42·11	52·63	00·00	94·74	1·47
TOTAL.	1,054	6	272	566	210	1,048	25·81	53·70	19·92	99·43	1·94

TABLE II.

Comparative incidence of gingivitis, calculus and plaques.
(Queen Mary College, Lahore—Girls.)

Age in years.	Number of subjects in group.	Number with no gingivitis.	Number with gingivitis.			Gingivitis figure.	Number with no calculus.	Number with calculus.			Calculus figure.	Number with no plaques.	Number with plaques.			Plaques figure.
			+	++	+++			+	++	+++			+	++	+++	
6-8	61	16	38	7	...	0·85	58	3	...	...	0·05	5	50	6	...	1·02
9-10	28	10	12	5	1	0·89	24	3	1	...	0·18	...	26	2	...	1·07
11-12	40	11	19	6	4	1·08	29	5	4	2	0·48	...	33	7	...	1·18
13-14	61	11	29	12	9	1·31	33	19	8	1	0·62	2	47	11	1	1·18
15-16	27	7	8	6	6	1·47	13	8	2	4	0·89	1	18	7	1	1·30
17-18	32	10	9	6	7	1·31	17	9	4	2	0·72	5	23	4	...	0·97
19-20	12	4	3	5	...	1·08	5	5	2	...	0·75	2	8	2	...	1·00
TOTAL	261	69	118	47	27	1·12	179	52	21	9	0·43	15	205	39	2	1·11

TABLE III.

Gingivitis incidence. Comparison of girls and boys (6 to 11 years).

(Queen Mary College, Lahore.)

	Age in years.	Number of subjects in group.	Per cent with no gingivitis.	Number with gingivitis.				Gingivitis figure.	Number with no calculus.	Number with calculus.			Calculus figure.	Number with no plaques.	Number with plaques.			Plaques figure.
				‾	++	+++	++++			+	++	+++++			+	++	++++	
Girls	6–7	39	33·33	22	4	...	...	...	39	...	...	...	...	4	32	3	...	...
	8–9	38	21·05	24	5	1	...	...	33	5	...	...	...	1	33	4	...	...
	10–11	29	34·18	11	7	1	...	...	23	3	2	1	...	...	23	6	...	...
	Total.	106	29·25	57	16	2	...	0·90	95	8	2	1	0·14	5	88	13	...	1·08
Boys	6–7	28	32·14	15	4	...	...	...	27	1	...	...	...	2	18	6	2	...
	8–9	23	8·70	11	10	...	...	...	12	8	3	...	...	2	15	6	...	...
	10–11	7	28·57	4	1	...	...	...	6	1	...	...	...	...	3	4	...	...
	Total.	58	22·41	30	15	...	...	1·03	45	10	3	...	0·28	4	36	16	2	1·28

disease figures being 77·59 per cent and 1·03 for the boys and 70·75 per cent and 0·90 in the case of the girls.

From the accumulated data of previous investigations in this country and from the figures here produced it is evident that the incidence of gingival disease is extremely high in all sections of the population. The disease is alarmingly prevalent not only in famine and deficient areas but also in apparently well-nourished population groups. Children in rural and urban areas alike are affected, and the disease is by no means confined to sections of the community of a low socio-economic level. In this country periodontal disease presents a problem infinitely more serious than dental caries to which an immunity of as high as 74 per cent has been reported in some areas (Day, 1944). From the viewpoint of its universally high incidence and potentialities for harm the problem of periodontal disease should command much more interest than in the past.

A more precise study of certain aspects of gingival disease and related factors has been made on a small group (121) of Islamia High School boys with the aid of the gingivitis diagnosis Chart already referred to (*see* end of paper). This was done in order to test the efficiency of methods used in other countries for assessing gingival health and disease and, if necessary, to modify and adapt those methods to Indian conditions in preparation for future and more comprehensive investigations along similar lines. It was expected that such a course might reveal certain productive avenues for further investigation.

(*iii*) *Variations in the colour of the gum.*—In view of the common use of tissue colour changes in the differentiation of health from disease, particular attention was given to the colour of the gum tissue. In the European there is considerable variation in colour even in apparently healthy gums which makes the use of colour change as a diagnostic aid of uncertain value. In the Indian the situation is further complicated by the presence in a high percentage of cases of a condition of a general epithelial pigmentation ranging from brown to black in colour, or alternatively and more commonly of patches of this pigmentation, sometimes extending as far as the interdental papillæ. It was found that 58 per cent of the subjects examined presented this anomaly in one form or the other. Investigation showed that though the correlation was not constant for individuals the incidence of gum pigmentation was in direct relation to the colour of the facial complexion or degree of skin pigmentation.

TABLE IV.

Gum pigmentation.

Facial complexion.	Number of subjects.	Number with no pigmentation.	Number with general pigmentation.	Number with patches of pigmentation.	Percentage with pigmentation.
Light-brown ...	6	6	0	0	0
Medium-brown ...	105	43	2	60	59
Dark-brown ...	10	2	0	8	80
TOTAL ...	121	51	2	68	58

Table IV shows that of those with light-brown, medium-brown and dark-brown skins, 0, 59 and 80 per cent respectively had one or other form of gum pigmentation superimposed on the usual pink colour. This anomaly occurring in almost three-fifths of the subjects examined, together with a greater variation in ' normal ' colour tones, not only minimizes the value of colour changes as criteria for gingival disease in India but also makes the utilization of pressure recovery tests of very doubtful value. Despite these observations a record was kept of colour changes where these were detectable. Of the total, 44·63 per cent showed discernible colour changes and 24·79 per cent were charted as doubtful. The most that can be said in view of the confusion created by these anomalies is that the incidence of detectable colour change was in direct proportion to the severity of the disease.

(*iv*) *Gingival pockets.*—The occurrence of periodontal pockets of varying depth was a universal finding in all cases of gingival disease. It is difficult to know just what constitutes a normal gingival crevice and this has not been standardized for Indian mouths. The location of the gingival crevice largely depends on the age of the individual. Up to the age of adolescence the bottom of the crevice is usually located on the surface of the enamel. Later, it is at or near the cemento-enamel junction and after middle age it is as a rule on the root surface (Kronfeld, 1940). In the hypertrophic type of gingivitis the cemento-enamel junction is not ordinarily exposed and this was the case in the subjects examined. In each case a record was kept of pocket depth measured from the base of the gingival crevice to the free margin of gum. In Table V is seen the average pocket depth in mm. in the labial, lingual and interdental regions in both upper and lower jaws. In all degrees of gingivitis the average pocket depth was significantly greater on the labial than on the lingual surface, the interdental pockets being of still greater depth. In most cases the depth of pocket was significantly greater in the upper than in the lower. Only in cases where there was a specific local cause such as deep overbite was the pocketing greater on the palatal than on the labial surface. Suppuration was recorded in only 4·13 per cent of the cases examined.

TABLE V.

Gingival pocket depth.

	Degree of gum disease.	Number of subjects.	Lateral pockets (average depth in mm.).	Lingual pockets (average depth in mm.).	Interdental pockets (average depth in mm.).
Upper	Slight to moderate	31	2·07	1·62	2·60
	Severe ...	66	2·40	1·88	2·92
	Very severe ...	19	2·74	2·05	3·21
Lower	Slight to moderate	35	1·93	1·29	2·27
	Severe ...	63	2·38	1·83	2·69
	Very severe ...	21	2·76	1·95	3·24

(*v*) *Hypertrophy of gum margins and papillæ.*—The thickening of the free margin of gum characteristic of the proliferative type of hypertrophic gingivitis was an almost universal occurrence in the large group of Islamia High School boys and in all those affected with the disease in Queen Mary College. The degree of hypertrophy was recorded as one, two or three *plus* signs and a hypertrophy figure arrived at by summating the *plus* signs. The average figure for the group was obtained by dividing by the number of subjects examined. The average for the interdental papillæ (1·99 in both upper and lower) was appreciably higher than that for the marginal crests (1·92 in the upper and 1·83 in the lower). The average figure for marginal hypertrophy in the lower marginal crests may possibly be related to the differences in salivary and serumal calculus deposition in the upper and lower. The higher figure for the interdental papillæ reflects the greater degree of papillary congestion and œdema. It was noted that where congestion or œdema did not occur there was not the same tendency for loss of the normal stippled or matt appearance of the epithelial surface. The regularity of the heaping up of tissue in the proliferative type, uncomplicated by œdema or congestion, would account for the retention of a more normal surface texture.

(*vi*) *Hæmorrhage.*—The presence or absence of hæmorrhage on firm digital pressure has frequently been used as a diagnostic criterion for gum disease. Table VI shows the findings in this investigation. Bleeding could be induced on digital pressure in 29 per cent of the upper and in 37 per cent of the lower gingivæ. The percentage showing hæmorrhage was in direct relation to the severity of the disease, but the value of this test as a diagnostic aid must be largely discounted in view of the fact that of the cases with gum disease approximately 70 per cent of the upper and over 60 per cent of the lower gingivæ showed no hæmorrhage. Moreover, it will be seen that as high as 12 per cent of lower gingivæ with slight to moderate gingivitis showed hæmorrhage, whereas in 26 per cent of those with very severe gum disease hæmorrhage could not be induced.

TABLE VI.

Hæmorrhage.

Degree of gingivitis.	UPPER GINGIVÆ.		LOWER GINGIVÆ.	
	Average hæmorrhage figure.	Per cent with hæmorrhage.	Average hæmorrhage figure.	Per cent with hæmorrhage.
Slight to moderate ...	0·09	9	0·12	12
Severe ...	0·26	26	0·44	41
Very severe ...	1·11	79	1·05	74

(vii) The incidence of salivary and serumal calculus in relation to gingivitis.— In recording the incidence of tartar deposits a differentiation was made between salivary and serumal calculus. The degree of deposit was expressed in each case as $+$, $++$ and $+++$ (slight, moderate and severe) and a ' calculus figure ' calculated by totalling the *plus* signs and dividing by the number of subjects in the group.

The results for a section of Islamia High ' School boys are set out in Table VII. Salivary calculus occurs predominantly in the lower jaw and serumal calculus in the upper. On comparing the frequency and degree of tartar deposits, as indicated by the calculus figure with the severity of gum disease, it is seen that, for subjects with (*a*) salivary calculus, (*b*) serumal calculus, and (*c*) both salivary and serumal calculus, the calculus figure increases with the severity of the disease in both upper and lower jaws. This relationship is maintained if the total calculus figure is considered. Also the percentage of the subjects with calculus significantly increases with the severity of the disease.

On the other hand it will be seen that in the upper, 64·71 per cent of subjects with slight to moderate gum disease and 43·94 per cent of those with severe disease were entirely free of calculus deposit. The association of gum disease with freedom from deposits was also noted in the lower but to a less extent.

Similarly, in the Queen Mary College, of the 261 girls examined 179 or 68·58 per cent showed a complete absence of calculus, while only 69 or 22·22 per cent were free from gingivitis.

It is commonly thought that calculus deposit is one of the most important factors in the production of gum disease. Cogan (1939) states that ' the most prevalent cause of gingivitis is the presence of calcareous deposits about the necks of the teeth '. From the above figures it is evident that while there may be an association between the presence of calculus and gum disease it is not an important ætiological factor in the type of gingival disease encountered in the present investigation.

No definite relation between calculus deposits and age could be established in the Islamia High School boys, but in the Queen Mary College girls there was a steady increase in the calculus figure with increase in age from 6 to 16 years, as also in the gingivitis figure (Table II).

The relative incidence of the various types of calculus expressed as a calculus figure was as follows :—

	Upper.	Lower.
Salivary calculus	0·20	1·07
Serumal calculus	0·48	0·02
Both salivary and serumal calculus ...	0·02	0·28

The total calculus figure in the lower (1·37) was more than twice that of the upper (0·62) and the percentage of subjects free from calculus was 8·26 in the lower and 43·80 in the upper despite comparable gum disease figures (Table VII). Absence of tartar on the palatal surface of the upper anterior teeth was a constant finding.

TABLE VII.

Incidence of salivary and serumal calculus in relation to gingivitis.

Gingivitis degree.	Number of subjects.	Number free of calculus.	Per cent free of calculus.	Number with salivary calculus. +	++	+++	Total with salivary calculus.	Per cent with salivary calculus.	Salivary calculus figure.	Number with serumal calculus. +	++	+++	Total with serumal calculus.
Upper													
Nil ...	2	2	100	...	...	...	...	0·00	...	...	...	...	...
Slight to moderate ...	34	22	64·71	3	...	...	3	8·82	0·09	8	1	...	9
Severe ...	66	29	43·94	12	2	...	14	21·21	0·24	17	3	1	21
Very severe ..	19	...	0·00	3	1	...	4	21·05	0·26	8	7	...	13
TOTAL ...	121	53	45·80	18	3	...	21	17·36	0·20	33	11	1	45
Lower													
Nil ...	2	...	0·00	2	...	...	2	100	1·00	...	...	...	...
Slight to moderate ...	35	9	25·71	18	2	...	20	57·14	0·63	1	...	...	1
Severe ...	63	1	1·59	29	17	3	49	77·78	1·14	2	...	...	2
Very severe ...	21	...	0·00	...	9	5	14	66·67	1·57	...	...	...	...
TOTAL ...	121	10	8·26	49	28	8	85	70·25	1·07	3	...	...	3

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Nil ...	0·00	...	0·00	...	00·00	...	0·38
Slight to moderate ...	26·47	0·29	0·00	...	35·29	12	0·60
Severe ...	31·82	0·39	3·03	0·05	56·06	37	1·42
Very severe ...	78·95	1·16	0·00	...	100	19	...
TOTAL ...	37·19	4·0	1·65	0·02	56·20	68	0·52
Nil ...	0·0	...	0·00	0·17	100	2	0·33
Slight to moderate ...	2·86	0·03	14·29	0·27	74·29	36	1·44
Severe ...	3·17	0·03	17·16	0·52	86	62	2·10
Very severe ...	0·00	...	33·33	...	100	21	...
TOTAL ...	2·48	0·02	19·01	0·28	91·74	111	1·37

(viii) Plaques and débris.—A figure for plaques and food débris was calculated in the same way as for tartar deposits. In the case of the Islamia High School boys the findings (Table VIII) indicate a definite tendency for both the plaques and débris figures to increase with the severity of the gum disease. In Queen Mary College girls the figure for plaques only was recorded and this was found to be in direct relation to the gingivitis figure and to increase with age from 6 to 16 years. Débris was present in 87 per cent of subjects in the upper and 86 per cent in the lower. Plaques were even more prevalent. The evidence was not sufficient to draw any definite conclusions regarding their importance as other than only contributing causative factors.

TABLE VIII.

Incidence of débris and plaques.

Extent of gingival disease.		Number of subjects.	Débris.				Débris figure.	Plaques.				Plaques figure.
			Nil.	+	++	+++		*Nil.*	+	++	+++	
Upper	*Nil*	2	2	...	...	...	0·00	1	1	...	...	0·50
	Slight to moderate	34	10	23	1	...	0·70	6	24	4	...	0·94
	Severe ...	66	4	47	14	1	1·18	...	50	15	1	1·26
	Very severe ...	19	...	14	4	1	1·32	...	12	7	...	1·37
	TOTAL ...	121	16	84	19	2	1·06	7	87	26	1	1·17
Lower	*Nil*	2	2	...	...	...	0·00	...	1	...	...	1·00
	Slight to moderate	35	11	21	3	...	0·77	6	26	4·	...	0·94
	Severe ...	63	4	46	12	1	1·16	...	44	18	1	1·32
	Very severe ...	21	...	16	4	1	1·29	...	9	10	2	1·67
	TOTAL ...	121	17	83	19	2	1·05	6	80	32	3	1·26

Data was recorded relative to tooth cleansing habits. In most cases cleansing was carried out very irregularly and it was felt that not much reliance could be placed on the information obtained. Questioning elicited the fact that 32 per cent never cleaned their teeth, 47 per cent used the teased twig (*dattan*) method, 4 per cent used a brush and 17 per cent other methods such as charcoal with the finger. The gingivitis figure was 1·90, 1·74, 2·00 and 2·00 respectively.

(ix) Dental caries in relation to gum disease.—Dental caries and faulty restorations are considered by some to be important factors in the production of gingival disease through irritation and injury to the gingivæ. In the group studied

in this connection no fillings or other restorations and no carious cavities were found in the anterior teeth and therefore could not be causative factors of disease in the regions examined. Cavities in posterior teeth could not be considered as causative agents except in so far as they contribute to general oral malhygiene. These findings are recorded in Table IX :—

TABLE IX.

Dental caries and gingivitis.

Degree of gum disease.	Number of subjects.	Number caries-free.	Number of teeth.	Number of cavities.	Number of cavities per child.	Per cent teeth carious.	Caries index.
Nil	2	0	56	6	3·00	10·71	0·11
Slight to moderate	34	8	893	130	3·82	14·56	0·15
Severe ...	66	22	1,807	211	3·20	11·68	0·12
Very severe ...	19	6	537	67	3·52	12·48	0·13
TOTAL ...	121	36	3,293	414	3·42	12·57	0·13

In the 3,293 teeth of the 121 subjects in the Islamia High School of which caries records were kept only 414 cavities were found, all in the posterior teeth. Of the total number of teeth only 12·57 per cent were carious or an average of only 3·42 per subject examined. It is seen that no relation exists between dental caries and the extent of gingival disease.

(*x*) *Malocclusion and gingivitis.*—Malocclusion is often referred to as an important factor in the ætiology of gingival disease, in view of the localized trauma it is said to produce on the periodontal tissues and the increased food impaction and calculus formation induced by dental irregularities.

In the group examined, comprising 121 Islamia High School boys, 54 showed a condition of normal occlusion and 67 had malocclusion. Of these, 53 and 66 respectively had gingivitis and the average gingivitis figure was 1·82 for the normal subjects and 1·87 in the case of those with malocclusion. For cases showing evident crowding the figure was 1·86 and for those with close bite 1·87. It would, therefore, seem that in the group examined malocclusion could not have played any more than a very minor rôle in the production of gingivitis ; nor was there any evidence to indicate that traumatic or pathologic occlusion was concerned in the disease condition.

SUMMARY AND CONCLUSIONS.

1. Using precise diagnostic criteria gingival conditions were investigated in 1,377 Indian children and adolescents of both sexes and different socio-economic levels.

2. Reference is made to the prevalence of various types of periodontal disease in India and its importance as a possible ætiological or contributing factor in systemic disorders.

3. An extremely high incidence of gingivitis, predominantly of the proliferative hypertrophic type, is reported, varying from 99·43 to 73·73 per cent at different social levels, with a tendency towards a higher incidence amongst males.

4. The diagnostic criteria for gum disease and methods of assessment are discussed in some detail with particular reference to Indian subjects.

5. Reference is made to difficulties in establishing a ' normal ' for gum colour due to wide variations and the presence of pigmentation in a high percentage of subjects. While not constant for individuals, the incidence of gum pigmentation was in direct relation to the degree of skin pigmentation.

6. The incidence of salivary and serumal calculus is discussed. Reasons are given for the conclusion that though there may be an association between the presence of tartar and gum disease it is not an important causative factor in the type of gingival disease encountered in the present investigation.

7. There was no evidence that oral hygiene in terms of plaques, débris and tooth cleansing habits played a major rôle in the ætiology of hypertrophic gingivitis.

8. In view of the absence of dental caries in the anterior teeth of the group studied in this connection, it is evident that caries could not be a causative factor of gingival disease.

9. The investigation disclosed no evidence to support the view that malocclusion is an important factor in the ætiology of hypertrophic gingivitis or that pathologic occlusion was concerned in the production of the disease.

10. In view of the above findings it would seem evident that the high incidence of hypertrophic gingivitis of the proliferative type reported in the present investigation is not due to the local irritating factors usually advanced as important causative agents, though these may have contributed in a greater or less degree to incidence or extent of lesions. It would seem more probable that the ætiology is mixed and that the condition may have a background of systemic predisposing factors such as mineral or vitamin deficiencies and metabolic or endocrine disturbances.

No positive evidence could be obtained in an earlier experiment designed to study the effect of ascorbic acid on gingival conditions (Day and Shourie, 1943). Mellanby and King (1934) have demonstrated the production of epithelial hyperplasia of the periodontal tissues through vitamin A deficiency. Associated with a very high incidence of hypertrophic gingivitis in the Punjab we find that the amount of vitamin A in the diets usually consumed is inclined to be low. Cases of Bitot's spots, dryness of the skin (varying from ordinary dryness to phrynoderma), the frank expressions of vitamin A deficiency, are encountered. Though this aspect of the problem has not yet been specifically investigated the conditions suggest that there may be an association between vitamin A deficiency and hypertrophic gingivitis. Also the Punjab diets are based on cereals and pulses and contain comparatively little milk, fruits and vegetables. There is, therefore, a possibility of an acid-ash imbalance.

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CHART.

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(Periodontal disease.)

NAME :   SEX :

AGE :   CLASS :

COMPLEXION : Facial.
                     Oral tissue.

Upper.	2	1	1	2
Caries				
Tartar				
Débris				
Plaques·				
Gingivitis				
(i) colour				
(ii) margin				
(iii) papilla				
(iv) enam.-cem. junction				
(v) texture				
(vi) pressure (a) recovery				
(b) hæmorrhage				
(vii) pockets (a) marginal, labial, lingual				
(b) interdental				
(viii) suppuration				
Lower. —	2	1	1	2
(viii) suppuration (b) interdental				
(vii) pockets (a) marginal, lingual, labial				
(vi) pressure (b) hæmorrhage				
(a) recovery				
(v) texture				
(iv) enam.-cem. junction				
(iii) papilla				
(ii) margin				
(i) colour				
Gingivitis				
Plaques				
Débris				
Tartar				
Caries				

Malocclusion.
Cleansing habits (a) Brush.   (b) *Dattan.*   (c) Other.
Total number of teeth.
Total number of cavities.

Miscellaneous notes :—

PLASMA PROTEINS IN HEALTH AND DISEASE.

Part I.

PLASMA PROTEINS IN HEALTHY INDIVIDUALS.

BY

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AND

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THE determination of plasma proteins in normal individuals has been carried out by several workers. Although there is general agreement among different workers with respect to the average normal concentration of each of the protein fractions, there would appear to be a wide margin between the lowest and the highest values found normally. Besides, these values for normal serum protein are not entirely satisfactory, since most of the studies have been made upon small groups of persons whose state of health in many cases is open to question. The difference in the technique in the analysis of proteins as used by different workers may also have led to certain discrepancies.

The largest series of plasma protein values reported in the literature, in which albumin and globulin of normal human blood were separated by the method of Howe (1921) and determined by the Kjeldahl procedure, are 32 analyses of plasma from 16 men and 16 women by Salvesen (1926); those of Linder, Lundsgaard and van Slyke (1924) from 8 normals, and those of Moore and van Slyke (1930) from 9 others; and 21 analyses of serum from 13 males and 8 females by Bruckman. d'Esopo and Peters (1930).

Gutman, Moore, Gutman. McClellan and Kabat (1941) have reported the results of the complete fractionation of serum proteins in 36 normal adults by the Howe's method with precautions to avoid the loss of filtrate protein through

adsorption on the filter-paper as shown by Robinson, Price and Hogden (1937). The results without these precautions during filtration are also recorded for 10 normal adults. By comparative analysis these authors have shown that the albumin level may be higher when such precautions are taken (as much as + 0·3 g. per cent).

There is, however, great paucity of data with respect to plasma protein values in normal Indians. Mudaliyar, Sundaram and Ramchandran (1933) reported the results of the analysis of serum proteins in 9 normals by the salting out method of Howe, followed by colorimetric method of Greenberg (1929). Lloyd and Paul (1928) presented data on 9 others by the refractometric method. The marked chemical, physiologic and immunologic differences between the different protein fractions of the blood plasma have made it increasingly important that a quantitative study be undertaken to determine the amount of albumin, globulin and fibrinogen present in the blood of healthy Indians. An accurate determination of the normal concentration of each of the separate protein fractions is essential for the interpretation of the results in pathologic conditions. This communication presents the results of determination of protein fractions in the blood plasma of 70 healthy individuals. The normal concentrations of 'euglobulin', and of 'pseudoglobulin' fractions of the total globulin have been included in this series, and form a baseline for our subsequent studies on the difference of disease on these' fractions of plasma protein.

MATERIAL AND METHOD.

The subjects of this study were mostly medical students and members of the staff. All subjects with good health records were taken as normals. Every attempt was made to exclude those who might present variations in any of the protein fractions as a result of malnutrition, mild or chronic infections. Six children included in this series were those who attended the hospital for slight injuries and were clinically declared as normal.

Most of the blood samples were taken from one to two hours after the usual breakfast. A few samples were taken an hour and a half after other meals. The blood samples were placed in a tube containing potassium oxalate in the concentration of 2 mg. per c.c. as an anticoagulant. The plasma was separated within an hour from the time the blood was taken.

The total protein was determined from the value of total nitrogen determined in 0·5 c.c. sample by the micro-Kjeldahl technique. The non-protein nitrogen was determined in 1 c.c. samples of plasma by the method of Folin and Wu (1919), and the difference between the total nitrogen and the non-protein nitrogen was multiplied by the conversion factor 6·25 to give the value of total protein. · The fibrinogen was determined in 0·5 c.c. of the plasma by precipitation as fibrin by the method of Cullen and van Slyke (1920), and the nitrogen was determined by taking the difference between the total nitrogen and the nitrogen in the fibrinogen-free filtrate.

The albumin and globulin were determined by the sodium sulphate method of Howe (*loc. cit.*). Absolute precautions to avoid a filtration error through adsorption on the filter-paper were not possible. For the determination of albumin

15 c.c. of 22·5 per cent solution of sodium sulphate (Merck's anhydrous) were added to 0·5 c.c. of the plasma and the mixture was allowed to remain at 37°C. for 3 hours. The precipitate of globulin was filtered through 9 cm. of Watman filter-paper. The first filtrate (approximately 2 c.c.) was discarded. The nitrogen was then determined in 10 c.c. of the last portion of the filtrate by the procedure indicated. The plasma globulin including the value of fibrinogen was determined by difference.

For the determination of '*euglobulin*', 15 c.c. of 14 per cent solution of anhydrous sodium sulphate were added to 0·5 c.c. of plasma and the mixture was allowed to stand at 37°C. for 3 hours. For filtration 7 cm. of Watman filter-paper (No. 50) was used. The nitrogen was determined in 10 c.c. of the last portion of the filtrate. The difference between the nitrogen in the fibrinogen-free filtrate and the nitrogen in the filtrate from 14 per cent sodium sulphate precipitation was taken as '*euglobulin*' nitrogen. The total protein and '*euglobulin*' were also determined in 0·5 c.c. of the serum for comparison.

RESULTS.

This series of 70 determinations of plasma proteins in normal healthy individuals consisted of 45 males and 25 females The age varied from 5 to 50 years. Most of the analyses were done during the rainy season extending from the end of June to the end of August. Only 12 out of 70 determinations were made in winter and the other 12 in the hot months of April, May and October. Hence a study of the influence of the season of the year on the concentration of plasma proteins was not possible. No significant difference in the value of plasma proteins was observed for the two sexes nor for groups of persons living on vegetarian and non-vegetarian diets.

In Tables I and II are included the results of fractionation of plasma proteins by the method of Howe in 45 males and 25 females respectively.

TABLE I.

Fractionation of plasma proteins by the method of Howe in 45 healthy males.

Number.	Age in years.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.
				(Results in g. per 100 c.c.)			
1	22	7·95	5·34	2·61	0·42	2·01	0·18
2	24	7·92	5·32	2·60	0·35	2·08	0·17
3	27	7·88	5·13	2·75	0·51	2·02	0·22
4	10	7·87	5·11	2·76	0·63	1·87	0·26
5	31	7·83	5·10	2·76	0·61	1·89	0·26

TABLE I—*contd.*

Number.	Age in years.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.
			(Results in g. per 100 c.c.)				
6	21	7·83	4·96	2·87	0·63	1·92	0·32
7	24	7·82	5·16	2·66	0·66	1·78	0·22
8	20	7·75	5·09	2·66	0·59	1·58	0·49
9	9	7·75	5·00	2·75	0·46	2·11	0·18
10	12	7·68	5·06	2·62	0·65	1·75	0·22
11	23	7·68	4·96	2·72	0·54	1·71	0·47
12	25	7·68	4·87	2·81	0·55	2·11	0·15
13	22	7·62	5·10	2·52	0·43	1·90	0·19
14	18	7·62	4·98	2·64	0·54	1·94	0·16
15	24	7·56	4·98	2·58	0·40	1·78	0·40
16	36	7·56	4·94	2·62	0·38	1·95	0·29
17	28	7·56	4·88	2·68	0·66	1·76	0·26
18	30	7·56	5·15	2·41	0·52	1·70	0·19
19	10	7·56	4·92	2·64	0·46	2·01	0·17
20	10	7·54	4·92	2·62	0·40	1·92	0·30
21	6	7·50	4·75	2·75	0·62	1·78	0·35
22	32	7·50	4·82	2·68	0·46	1·84	0·38
23	28	7·43	5·06	2·37	0·36	1·81	0·20
24	18	7·40	4·86	2·54	0·43	1·95	0·16
25	30	7·40	4·89	2·51	0·68	1·36	0·47
26	27	7·39	4·75	2·64	0·54	1·90	0·20
27	21	7·37	4·85	2·52	0·45	1·84	0·23
28	19	7·37	4·70	2·67	0·35	2·16	0·16
29	25	7·37	4·86	2·51	0·59	1·77	0·15
30	19	7·30	4·97	2·33	0·54	1·61	0·18
31	22	7·25	4·67	2·58	0·52	1·88	0·18

TABLE I—*concld.*

Number.	Age in years.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.
			(Results in g. per 100 c.c.)				
32	24	7·18	4·72	2·46	0·54	1·71	0·21
33	27	7·18	5·03	2·15	0·37	1·52	0·26
34	27	7·06	4·38	2·68	0·69	1·69	0·30
35	28	7·03	4·30	2·73	0·72	1·75	0·26
36	26	7·65	5·06	2·59			
37	30	7·50	5·05	2·45			
38	32	7·50	5·05	2·45			
39	32	7·46	4·75	2·71			
40	24	7·40	5·20	2·20			
41	22	7·38	5·08	2·30	Globulin subfraction not determined.		
42	23	7·31	4·94	2·37			
43	30	7·30	4·74	2·56			
44	25	7·25	4·87	2·38			
45	28	7·12	4·44	2·71			

		Number.	Mean, g. per 100 c.c.	Standard deviation.	Coefficient of variation.
Total protein	...	45	7·51	0·23	3·0
Albumin	...	45	4·93	0·20	4·0
Globulin	...	45	2·58	0·16	6·0
Euglobulin	..	35	0·52	0·10	20·0
Pseudoglobulin	...	35	1·84	0·17	9·6
Fibrinogen	..	35	0·25	0·09	39·0

TABLE II.

Fractionation of plasma proteins by the method of Howe in 25 healthy females.

Number.	Age in years.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.

(Results in g. per 100 c.c.)

Number.	Age in years.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.
1	25	7·95	5·20	2·75	0·70	1·70	0·35
2	19	7·93	5·18	2·75	0·78	1·78	0·19
3	20	7·87	5·01	2·86	0·52	2·08	0·26
4	30	7·87	5·01	2·86	0·45	2·24	0·17
5	23	7·81	5·15	2·66	0·65	1·79	·0·22
6	25	7·75	5·04	2·71	0·69	1·74	0·28
7	20	7·62	4·87	2·75	0·57	1·72	0·46
8	22	7·62	4·87	2 75	0·49	2·05	0·21
9	25	7·62	5·19	2·43	0·67	1·64	0·12
10	23	7·62	5·00	2·62	0·47	2·03	0·12
11	23	7·54	4·93	2·61	0·63	1·67	0·31
12	26	7·46	5·10	2·36	0·25	1·95	0·16
13	24	7·44	4·76	2·68	0·68	1·71	0·29
14	50	7·43	4·50	2·93	0·67	1·83	0·43
15	55	7·43	4·75	2·68	0·58	1·68	0·42
16	21	7·43	4·81	2·62	0·52	1· 9	0·21
17	25	7·37	4·87	2·5)	0·o9	1·48	0·33
18	22	7·31	4·69	2·62	0·70	1·68	0·24
19	31	7·31	5·00	2·31	0·66	1·48	0·17
20	26	7·25	4·69	2·56	0·70	1·60	0·26
21	23	7·18	4·62	2·56	0·55	1·53	0·48
22	22	7·18	4·67	2·51	0·36	1·90	0·25
23	26	7·12	4·73	2·39	0·68	1·49	0·22
24	36	7·06	4·86	2·20	0·57	1·45	0·18
25	24	7·00	4·75	2·25	0·58	1·46	0·21

	Number.	Mean, g. per 100 c.c.	Standard deviation.	Coefficient of variation.
Total protein	25	7·49	0·27	3·6
Albumin	25	4·87	0·21	4·0
Globulin	25	2·60	0·18	7·0
Euglobulin ...	25	0·58	0·12	21·0
Pseudoglobulin ...	25	1·74	0·21	12·3
Fibrinogen ...	25	0·26	0·09	35·0

TABLE III.

Plasma protein values in individuals apparently healthy but presenting variations in the value of different protein fractions due to malnutrition or as a result of mild or latent infection.

Number and sex.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudoglobulin.	Fibrinogen.	REMARKS. Age in years.	REMARKS. Weight in lb.

(Results in g. per 100 c.c.)

Group A.—Underweight.

Number and sex.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudoglobulin.	Fibrinogen.	Age in years.	Weight in lb.
1 M	6·87	4·46	2·41	0·63	1·58	0·20	25	96
2 F	6·50	4·60	1·90	0·38	1·21	0·31	23	85
3 M	6·62	4·31	2·31	0·61	1·52	0·18	27	103
4 M	6·56	4·55	2·01	0·32	1·47	0·22	26	100

Group B.—Associated infection.

Number and sex.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudoglobulin.	Fibrinogen.	Remarks.
5 M	7·62	4·76	2·86	1·10	1·58	0·18	Gets repeated attack of sore throat; hoarseness of voice.
6 M	7·31	4·60	2·71	1·34	1·04	0·33	Gets frequent attack of sore throat.
7 M	7·62	4·49	3·13	1·36	1·37	0·40	Gets repeated attack of malaria.
8 M	7·62	4·58	3·04	1·28	1·12	0·64	Gets attack of malaria.
9 M	7·37	4·63	2·74	0·82	1·45	0·47	Slight bronchitis.
10 M	7·68	4·83	2·85	0·92	1·27	0·66	Chronic gastritis.
11 M	7·81	4·82	2·99	1·33	1·37	0·24	Severe headache relieved on lumbar puncture.
12 M	7·93	4·72	3·21	0·87	2·13	0·21	Allergic dermatitis, for 3 years.
13 M	8·05	4·98	3·07	0·81	1·90	0·36	High blood pressure, high pulse rate.
14 M	7·50	4·69	2·81	1·00	1·81	Serum	Gets attack of œdema of legs. Blood pressure of normal. N.p.n. 35 mg. per 100 c.c.
15 M	8·93	4·45	4·48	1·69	1·92	0·87	Incubation of malaria. Had an attack of malaria 2 days after the blood was analysed.
16 M	7·26	4·07	3·19	1·07	1·72	0·40	Apparently normal when the blood was taken. After 20 days fell ill. Clinically tuberculosis of kidney.
17 M	7·58	4·22	3·36	0·92	2·44	Serum	Had an attack of pyelitis.

Total protein.—The mean value of total protein for 25 males is 7·51 g. per 100 c.c.. with a standard deviation of 0·23 g. and 7·49 g. per 100 c.c., with a standard deviation of 0·27 g. for 25 females. The range is from 7·1 g. to 7·93 g. per 100 c.c., more than 75 per cent of the results falling within the range of 7·2 g. to 7·89 g.

Albumin.—The range of albumin in this series is from 4·3 g. to 5·34 g. per 100 c.c.   The mean value of albumin for 45 males is 4·93 g. per 100 c.c., with a standard deviation of 0·20 and the mean value for 25 females is 4·87 g. per 100 c.c., with a standard deviation of 0·21.   Inspection of the results of Tables I and II reveals at once a most striking and unexpected uniformity of results, the significant

GRAPH.
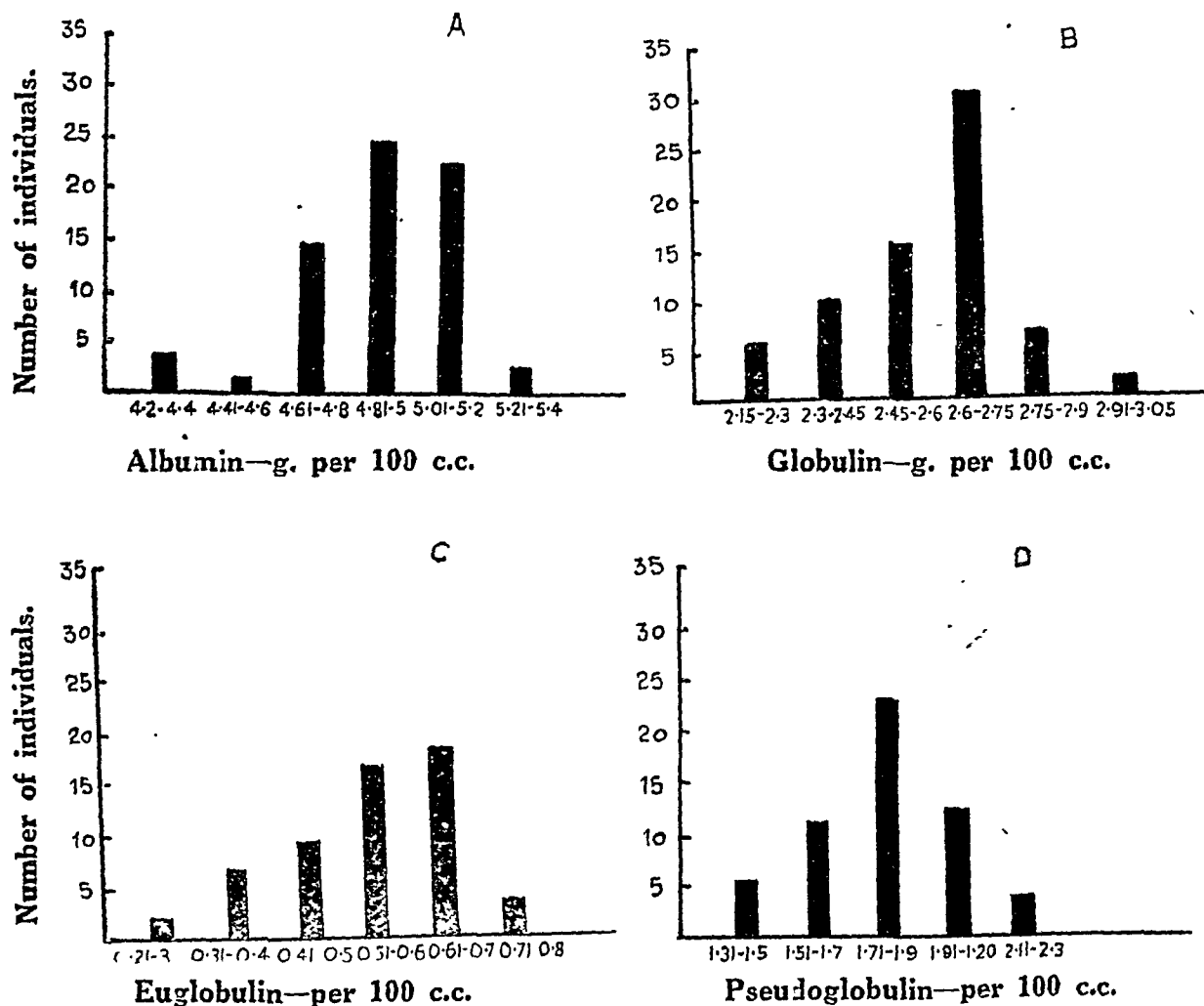
Frequency distribution of plasma proteins determined on 70 normal individuals (Indians).

variations being very few.   This is further illustrated in the Graph, fig. A, which shows that more than 85 per cent of the results lying within the narrow range of 4·6 g. to 5·2 g. per 100 c.c. and 40 per cent of them within the narrow limit of 5·0 g. to 5·3 g. per 100 c.c.

No significant difference in the value of albumin has been found between the sexes nor between groups of healthy members living on vegetarian and non-vegetarian diets.

Globulin.—The spread of the values of total globulin is from 2·15 g. to 2·93 g. per 100 c.c. with ¾ of the results falling between 2·2 g. and 2·68 g. The distribution of globulin values shows a definite skew towards the higher globulin concentration, especially in the case of females. It seems likely that a few individuals have been included in the series who were not actually ill when blood samples were taken but might have shown higher globulin as a result of mild infection.

Euglobulin.—The range of euglobulin is from 0·25 g. to 0·78 g. per 100 c.c. with a combined mean of 0·55 g. for both the sexes. The distribution of euglobulin values about the mean shows a skew towards a higher value somewhat similar to that observed with total globulin. The increased tendency towards a higher concentration of euglobulin is again prominent in females.

Total pseudoglobulin.—The spread of the values of pseudoglobulin in our series is from 1·36 g. to 2 24 g. per 100 c.c. with a mean of 1·84 g. in males and 1·74 g. in females, 48 values out of 60 falling in the range of 1·6 g. to 2·1 g. per 100 c.c.

Fibrinogen.—The mean value of fibrinogen for 35 males is 0·25 g. per 100 c.c. with a standard deviation of 0·095 g. and the mean for 25 females is 0·26 g. per 100 c.c., with a standard deviation of 0·093 g. The range is from 0·12 g. to 0·48 g., with more than 80 per cent of the results falling in the range of 0·15 g. to 0·40 g. per 100 c.c. This is in agreement with the general experience.

In Table III are included the results of fractionation of plasma proteins in 17 subjects who were apparently healthy but presented variations in the values of different protein fractions either due to malnutrition or as a result of mild, chronic or latent infection.

From the results of Table III it is evident that the values of albumin and globulin in all the subjects included in group A are in the lower range of normal, so that the total protein is also low and below 7 g. per 100 c.c. in every case. It is possible that in undernourished and otherwise healthy individuals, the protein reserve exists at a low level without any subjective or objective manifestation except that these subjects fail to attain the normal bodyweight. The lowered store of body protein is reflected in the low level of albumin and globulin. The low globulin in all the subjects was associated with a lowered range of pseudoglobulin, the euglobulin remaining normal. An inspection of the results of fractionation of serum protein in 36 normal individuals presented by Gutman *et al.* (*loc. cit.*) in Table I, series A, will show that a low level of pseudoglobulin can be seen only in those subjects whose total protein was below 6·6 g. per 100 c.c. The significance of such low pseudoglobulin in these cases is not properly understood.

The results of case 5 to case 11 of Table III are significant. In all the cases the concentration of albumin was within normal range, while the values of globulin were above the normal mean. In spite of the increased globulin, the pseudoglobulin was decidedly low in all the subjects, while the values of euglobulin show a definite increase. It is not suggested that the lowered resistance to infection as observed in these cases was due to the low pseudoglobulin content indicating the association of the antibodies to the pseudoglobulin fraction, but it is nevertheless a striking

fact that the pseudoglobulin was low in all who were either undernourished or had some form of mild or chronic infection.

The results of other cases included in Table III show that the globulin increment was due to an increase in both the euglobulin and the pseudoglobulin fractions, thus conforming with the characteristic pattern of chronic infection. Cases 15 and 16 are of special interest as the results clearly indicate that abnormality in the protein fractions becomes apparent long before the disease becomes clinically perceptible.

It is proposed that individuals showing any of the manifestations, such as loss of weight or strength, loss of stamina or a decreased resistance to infection, should have the blood proteins examined for any possible state of protein deficiency or for the possible presence of mild, chronic or latent infection.

DISCUSSION.

Many methods have been employed for the determination of plasma protein concentration. Doubtless many discrepancies in the literature are explained by the variation in the method used. The Kjeldahl procedure is preferable to others in that it leads to far fewer discrepancies and the results obtained are highly reproducible.

A variety of salts have been used by different workers to separate the albumin and globulin. At the present time, the sodium sulphate method of Howe (*loc. cit.*) is being widely used for the determination of the various components constituting the protein complex of the blood plasma. The terms albumin, globulin, euglobulin and pseudoglobulin have been applied not to any chemical entities but to the fractional part of the total protein separated by precipitation at specific salt concentration. The fraction precipitated by 22·5 per cent solution of sodium sulphate is commonly referred to as globulin and the protein remaining in solution as albumin. Only a small portion of the total globulin in normal human serum is precipitated by 14·5 per cent solution of sodium sulphate and is designated as euglobulin. The portion remaining in solution is known as pseudoglobulin.

Table IV gives a summary of the plasma protein values reported in the literature in which the sodium sulphate method was used for the separation of albumin and globulin. Lloyd *et al.* (*loc. cit.*) used ammonium sulphate for the separation of serum proteins.

The mean albumin concentration of 4·93 g. per 100 c.c. for 45 males and 4·87 g. for 25 females which we have found in our series is in agreement with the mean of 5·06 for males and 4·98 g. per 100 c.c. for females reported by Bruckman *et al.* (*loc. cit.*). The combined mean of 4·90 g. per 100 c.c. for the entire series of 70 cases is in absolute agreement with the mean of 4·87 g. for 10 adults reported by Gutman *et al.* (*loc. cit.*) under sub-heading B; but slightly lower than the mean of 5·2 g. per 100 c.c. for 36 normal adults included under sub-heading A. This slightly low value (about 0·3 g. per 100 c.c.) is possibly due to the loss of filtrate protein through adsorption by the filter-paper as explained by those authors. The range of albumin values in their series of 36 adults under sub-heading A—4·7 g. to 5·7 g.—is, therefore, somewhat higher than the range of 4·3 g. to 5·34 g. per 100 c.c. which we have found in this series.

TABLE IV.

Plasma protein values reported in the literature.

Author and date.	Number of cases.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.
			(Results in g. per 100 c.c.)				
Linder, Lundsgaard and van Slyke (1924).	7*	...	3·63–4·90	2·45–2·89	...	...	...
	...	6·72	4·11	2·61	...	...	...
Salvesen (1926) ...	16 M*	...	3·95–5·24	1·96–3·16	...	...	...
	...	7·02	4·44	2·68	...	...	...
	16 F*	...	3·77–4·80	2·18–3·55	...	...	...
	...	7·23	4·55	2·68	...	...	...
Moore and van Slyke (1930).	9*	...	4·0–4·5	2·8	...	...	...
	...	...	4·3	...	...	...	...
Bruckman, d'Esopo and Peters (1930).	13 M†	...	4·37–5·65	1·32–2·92	...	...	...
	...	...	5·06	1·89	...	...	...
	8 F†	...	4·71–5·17	2·02–3·22	...	...	...
	...	...	4·98	2·62	...	...	...
Lloyd and Paul (1928).	10†	...	4·31–5·06	2·76–3·23	0·14–0·22	...	...
	...	7·52	4·59	2·93	0·16	2·77	...
Stacey (1945) ..	12†	...	3·6–5·5	2·2–3·3	0·18–0·59	1·7–3·1	...
	...	7·57	4·73	2·84	0·38	2·40	...
Mudaliyar, Sundaram and Ramchandran (1933).	9†	...	3·5–5·0	1·15–2·65	0·25–1·25	0·8–2·4	...
	...	6·12	4·02	2·10	0·60	1·50	...
Gutman, Moore, Gutman, McClellan and Kabat (1941).	36†	7·2	4·7–5·7	1·3–2·5	...	...	Series A
	...		5·2	2·0	0·20	1·80	
	10†	7·2	4·6–5·2	1·9–2·7	0·18–0·60	...	Series B
			4·87	2·31	0·39	1·88	
Present series (1947)	45 M*	7·51	4·30–5·34	2·15–2·87	0·35–0·72	1·36–2·16	0·25
	...		4·93	2·58	0·52	1·84	
	25 F*	7·49	4·37–5·20	2·20–2·93	0·25–0·78	1·45–2·24	0·26
	...		4·87	2·60	0·58	1·74	

* Plasma.   † Serum.

The albumin fraction is readily affected by the dietary intake of protein, but cannot be increased beyond the usual normal limit by excessive protein feeding: As yet, we do not know if there is an upper threshold for albumin concentration for normal individuals, if so, how constant it is. A knowledge of upper threshold is obviously of great clinical significance.

The values of albumin reported in the literature fall in the range of 3·6 g. to 5·4 g. per 100 c.c.; values as high as 5·65 g. to 5·7 g. have, however, also been reported. From the frequency of distribution of values for albumin it appears that all values below 4 g. per 100 c.c. are decidedly below the normal healthy level and the value of 5·7 g. per 100 c.c. may be the extreme limit in normal adults. In all probability the concentration of albumin will not usually exceed 5·4 g. per 100 c.c. in average healthy individuals.

For the entire group of 70 adults the mean globulin concentration is about 2·59 g. per 100 c.c. This mean value is essentially the same as those given in Table IV, as reported by Linder *et al.* (*loc. cit.*) and by Salvesen (*loc. cit.*) and also is in relatively good agreement with the mean serum globulin value of 2·31 g. per 100 c.c. reported by Gutman *et al.* (*loc. cit.*) for their 10 normal adults under B. In general, the range of serum globulin values reported under sub-heading B—1·9 g. to 2·7 g. per 100 c.c.—falls within the range of values covered by our series—2·2 g. to 2·9 g. per 100 c.c. (including the values of fibrinogen). The distribution of our values shows a skew towards the higher globulin concentration, as shown in the Graph, fig. B. From the results of Table III it appears that values of globulin above 2·8 g. per 100 c.c. is an evidence for an organic disease process.

Fewer studies on sub-fractionation of globulin in the blood plasma of normal adults have been made so far. The mean value of euglobulin reported by different workers for their respective small group of individuals vary widely with respect to each other. The lack of agreement may be ascribed to (1) wide range of normal variation or (2) difference in the technique employed. Mudaliyar *et al.* (*loc. cit.*) and Stacey (1945) employed colorimetric method for the determination of euglobulin. The values of euglobulin reported by Gutman *et al.* (*loc. cit.*) were determined by the method of Howe followed by micro-Kjeldahl technique.

The mean value of 0·55 g. per 100 c.c. which we have found for the entire series of 70 normal adults is in good agreement with the mean value of 0·6 g. reported by Mudaliyar *et al.* (*loc. cit.*) for 10 normal Indians. On the other hand, our mean is somewhat higher than the mean of 0·39 g. per 100 c.c. reported by Stacey (*loc. cit.*) and distinctly higher than the mean of 0·2 g. reported by Gutman *et al.* (*loc. cit.*) for their 36 normal adults under sub-heading A. In spite of our higher mean most of the values are well within the range of 0·18 g. to 0·6 g. per 100 c.c. reported by these authors. There is, therefore, a suggestion of essential constancy, upset in a certain proportion of cases by interfering factors, such as mild or chronic infection or recent immunization, etc. Besides, a difference of 0·2 g. per 100 c.c. in normal serum becomes imperceptible in conditions causing hyperglobulinemia.

The range of total pseudoglobulin in our series is from 1·3 g. to 2·24 g. per 100 c.c., with a mean of 1·84 g. per 100 c.c. for 35 males and 1·74 g. for 25 females. Our mean is in good agreement with the mean of 1·8 g. per 100 c.c. reported by

Gutman *et al.* (*loc. cit.*) for 36 normal adults in their series A. The spread of their values is somewhat wider varying in the range of 1·1 g. to 2·2 g. per 100 c.c. All values below 1·4 g. per 100 c.c. are seen only in those cases in which the total protein was below 7·0 g. per 100 c.c. We are led to believe that all values below 1·4 g. per 100 c.c. are subnormal. This is also evident from the results of our Table III. which show that persons who are underweight or those who constantly suffer from common cold, sore throat or manifest signs of mild infection exhibit low pseudo-globulin varying in the range of 1·04 g. to 1·58 g. per 100 c.c.

The range of fibrinogen in this series is from 0·12 g. to 0·48 g. per 100 c.c.. with a mean of 0·25 g. for 35 males and 0·26 g. per 100 c.c. for 25 females. This mean is in perfect agreement with the mean of 0·25 g. per 100 c.c. reported by Ham and Curtis (1938) for 193 determinations on adults and in the work reviewed by them on plasma fibrinogen in normal persons. The range of fibrinogen reported by these authors is from 0·19 g. to 0·38 g. per 100 c.c. Our values are somewhat wider including values below those found in the literature and a few which are higher, more than 80 per cent of the values lying in the range of 0·16 g. to 0·39 g. per 100 c.c.

The spread of total protein in this series is from 7·0 g. to 7·95 g. per 100 c.c.. with more than 80 per cent of the values lying between 7·3 g. to 7·95 g. This is in agreement with general experience.

Determination of total protein without separate analysis of the different protein fractions may not indicate the true status: inasmuch as a diminution of serum albumin may co-exist with a corresponding increase in serum globulin so that the total protein will remain normal. The function of plasma proteins may be attributed primarily to one or the other fraction rather than to their total concentration. The albumin fraction is the one of considerable significance, first because it is the only fraction which is characteristically affected ; and second. because of its importance in maintaining the colloid osmotic pressure of the blood plasma, which controls the water balance of the body. The globulin fraction on the other hand is concerned with the immune bodies. It is essential that the absolute values of albumin, globulin and fibrinogen should be recorded for the sake of clarity.

The expression albumin : globulin ratio, without reference to the total protein is of little or no significance. The interpretation of results of pathologic conditions is based on the concentration of each of separate protein fractions rather than to a ratio of any two components.

SUMMARY.

1. The results of fractionation of plasma proteins by the method of Howe are recorded in 70 healthy individuals. The series consists of 45 males and 25 females varying in age from 5 to 50 years. The spread and the mean in g. per 100 c.c. are : For total protein. 7·0 g., to 7·95 g. with a mean of 7·51 g. for males and 7·49 g. for females : for albumin, 4·3 g. to 5·34 g.. with a mean of 4·93 g. for males and 1·87 g. for females ; for globulin, 2·12 g. to 2·9 g.. with a mean of 2·58 g. for males and 2·60 g. in females. The range of euglobulin was from 0·25 to 0·78 g. per 100 c.c., with a mean of 0·55 g. per 100 c.c.

2.   The spread of the values of total pseudoglobulin is from 1·3 g. to 2·24 g. with a mean of 1·84 g. for males and 1·74 g. per 100 c.c. for females. The mean value of fibrinogen is 0·25 g., with a range from 0·12 g. to 0·48 g. per 100 c.c.

3.   No significant difference in the value of any of the protein fractions was found between the two sexes, nor between groups of healthy persons living on vegetarian and non-vegetarian diets.

4.   Results of fractionation of plasma proteins are recorded in 17 apparently healthy individuals who presented variations in the value of globulin fractions either due to malnutrition or as a result of mild or chronic infection. There are indications that abnormality in the protein fractions becomes apparent long before the disease is clinically perceptible.   It is proposed that individuals showing any of the manifestations, such as loss of weight, decreased resistance to mild infections such as common cold, sore throat, etc., should have the blood proteins determined for any possible state of protein deficiency or for the possible presence of some mild or chronic infections.

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PLASMA PROTEINS IN HEALTH AND DISEASE.

Part II.

FRACTIONATION OF PLASMA PROTEINS IN HYPERPROTEINÆMIA.

BY

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(*An Inquiry under the Indian Research Fund Association.*)

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HYPERPROTEINÆMIA is almost invariably due to an increase in globulin, since an increase in the concentration of albumin in blood plasma is seldom encountered. Hence, as stated by Kagan (1943), 'a study of hyperproteinæmia is essentially a study of hyperglobulinæmia'.

Recent literature indicates that hyperproteinæmia may not be such a rare occurrence as was once believed. Earlier, Jeghers and Selesnick (1937) reviewed the subject of hyperproteinæmia and reported 13 cases in which serum protein was above 8 g. per 100 c.c. Bing (1937) found 7 hyperglobulinæmic serum among 3,697 serum examined by the routine 'formol-gel' test. Gutman *et al.* (1941) reported the results of fractionation of serum protein in 8 cases of lymphogranuloma venereum, 3 cases of sarcoid, 20 cases of multiple myeloma and 4 cases of miscellaneous infections presenting hyperglobulinæmia. Cardon and Atlas (1943) presented 54 cases in which the value of total protein was above 8·5 g. per 100 c.c. Kagan (*loc. cit.*) presented 50 cases of hyperglobulinæmia due to 16 different causes. He considered hyperglobulinæmia to be present when the serum globulin was above 3 g. per 100 c.c. or when, in the absence of a globulin determination, the total protein was above 8 g. per 100 c.c. The determination of serum protein was done by a macro-Kjeldahl modification of Howe's (1921) procedure and the author's falling-drop technique. Of the total number of cases studied, 35 cases were found to have the values of total protein above 8 g. per 100 c.c.

Hyperglobulinæmia is likely to be more common in localities where kala-azar or lymphogranuloma venereum is prevalent. All studies indicate that lymphogranuloma venereum is much more frequent in Negroes. Beeson and Miller (1944)

found hyperglobulinæmia in 5·6 per cent of males and 8·3 per cent of females in Negro population of Georgia. Other diseases which exhibit an elevation of serum globulin do not appear to manifest such racial affinity and none of them is so common as to affect 5 to 8 per cent of the general population at one time. There is little information available regarding the general incidence of hyperglobulinæmia in this country.

The present report deals with 73 cases of hyperglobulinæmia associated with hyperproteinæmia. These cases in which the total protein was above 8 g. per 100 c.c. were found in the examination of blood plasma obtained by random sampling from more than 1,500 patients suffering from a large variety of diseases and may, therefore, represent a true general incidence of hyperproteinæmia. The data were collected over a period of four years.

A study of the literature reveals that hæmoconcentration resulting from dehydration is one of the commonest causes of hyperproteinæmia. As the determination of blood volume by the dye method is elaborate and time consuming, the interpretation of the state of dehydration in the present studies was based on clinical observation.

The fractionation of plasma proteins was done by the method of Howe (*loc. cit.*). Fibrinogen was determined on oxalated plasma by the method of Cullen and van Slyke (1920). Complete fractionation of plasma proteins was carried out in an attempt to determine whether the increase in any of the globulin subfractions is in any way specific for any particular disease, and whether or not the alterations in the value of separate protein fractions can be correlated with the activity of the disease.

Hyperglobulinæmia and occasionally hyperproteinæmia have been found in a number of disorders. 'The most frequent cause of hyperglobulinæmia is an infection, and the increase of serum globulin has been placed in connection with the formation of antibodies.' Aside from its rôle in antibody formation almost nothing is known as to the origin of globulin.) Bing and Plum (1937) presented clinical evidence which suggest that globulin is formed by the plasma cells of the bone-marrow and by the cells belonging to the reticulo-endothelial system in and outside the marrow.

The exact nature of the globulin increase in various infections is not known. With the accumulation of our knowledge of their variation in different clinical conditions, it is expected that eventually there will come understanding of the factors and sites involved in the production of the various protein components.

RESULTS.

The results of fractionation of proteins in the blood plasma obtained from more than 1,500 patients indicate that there are only a few examples of conditions in which only albumin is affected, the globulin remaining within normal limits. On the other hand, there are a large group of conditions in which an increase in the value of globulin is observed at one time or another during the course of the disease. An increase in the value of globulin is almost invariably associated with a lowering of albumin, so that only occasionally is the increase in globulin so great that one can speak of hyperproteinæmia as well.

A classification of the cases presenting hyperproteinæmia with a summary of results obtained is shown in Table I. Data representative of the results of fractionation of proteins by the method of Howe in each disease will be treated separately.

TABLE I.

Classification of the cases of hyperproteinæmia with a summary of the results obtained.

Conditions.	Total number of patients studied.	Number presenting hyperpro-teinæmia.	Total protein varying between		Total globulin varying between		
			8 to 9	9 to 10	4 to 5	5 to 6	6 to 8
			g. per 100 c.c.		g. per 100 c.c.		
Leukæmia ...	4	3	3	0	4	0	0
Syphilis	30	12	12	0	10	0	0
Venereal lympho-granuloma.	14	12	4	8	4	8	2
Tubercular adenitis ...	3	3	3	0	2	0	0
Pulmonary tuberculosis	40	7	7	0	15	4	0
Kala-azar ...	2	2	0	2	0	0	2
Cirrhosis	56	10	6	4	22	5	4
Jaundice	32	2	2	0	3	0	0
Acute nephritis ...	74	6	4	2	10	2	0
Congestive cardiac failure.	36	5	5	0	6	1	0
Miscellaneous other conditions.	200	11	8	3	...	...	...

HYPERPROTEINÆMIA IN LEUKÆMIA.

Leukæmia is a disease involving bone-marrow. The monocytes are considered to be a part of the reticulo-endothelial system. In a case of monocytic leukæmia, reported by Kagan (*loc. cit.*), the total protein was 10·1 g. per 100 c.c. and the value of serum globulin varying from 4·3 g. to 6·7 g. According to him such marked increase in serum globulin, if found to be usual in this disease, would lend further support to the view that globulin is formed by the reticulo-endothelial system. There are also references of increased globulin in 2 cases of myeloid and 2 of lymphatic leukæmia, but in none of these cases the increase in globulin is as striking as in the case of monocytic leukæmia.

We have in this series 3 cases of lymphatic leukæmia and 1 of chronic myeloid leukæmia. The results of fractionation of plasma proteins are shown in Table II :—

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TABLE II.

Partition of serum protein in leukæmia.

</div>

Number.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	DIAGNOSIS.
	Results expressed in g. per 100 c.c.					
1	8·43	4·18	4·25	2·60	1·65	Chronic myeloid leukæmia.
2	8·36	3·67	4·69	1·85	2·84	Lymphatic leukæmia.
3	8·00	3·17	4·83	1·01	3·82	., ,,
4	6·65	2·45	4·20	1·08	3·12	,, .,

Hyperproteinæmia was present in 3 of the 4 cases studied. The concentration of total protein was, however, lower than the case of monocytic leukæmia reported by Kagan (*loc. cit.*). The globulin increment in all the 3 cases of lymphatic leukæmia was due to a marked increase in the pseudoglobulin fraction. A similar increase in the pseudoglobulin fraction was reported by Gutman *et al.* (*loc. cit.*) in 1 case of lymphatic leukæmia studied by them. The number of cases is too small to attempt to draw any definite conclusions.

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FRACTIONATION OF PLASMA PROTEINS IN SYPHILIS AND IN VENEREAL LYMPHOGRANULOMA.

</div>

It was the opinion of the earlier workers that syphilis produces a definite increase in the value of serum globulin. Lloyd and Paul (1928) studied 11 cases of secondary syphilis and reported that the concentration of globulin was above 3 g. per 100 c.c. in all but 3 cases. There was only 1 case in which the total protein was above 8 g. Wu (1922) reported 2 cases of syphilis with hyperproteinæmia. The occurrence of hyperglobulinæmia and hyperproteinæmia in uncomplicated syphilis has been questioned by Jones and Rome (1939). According to them the hyperglobulinæmia observed in these cases may be due to the presence of lymphogranuloma venereum. They did not find a marked increase in serum globulin in patients with syphilis except in those who had positive Frei test.

In lymphogranuloma venereum an elevation of serum globulin is an important feature of the disease. Jones and Rome (*loc. cit.*) studied 79 patients with lymphogranuloma venereum. The majority of their patients had the total protein above 8 g. per 100 c.c., the highest value obtained being 13·33 g. Kampmeier *et al.* (1939) found hyperproteinæmia in 62 of the 67 cases studied and considered this fact to be of diagnostic value. Schamberg (1940) found total protein above 8 g. per 100 c.c.

in 17 of the 20 patients with early lymphogranuloma venereum. The highest value of total protein reported by Cardon and Atlas (*loc. cit*) in their series of 6 cases was 11·44 g. per 100 c.c. and the highest value of globulin was 7·66 g. Hyperproteinæmia was present in 8 of the 11 cases reported by Kagan (*loc. cit.*).

Gutman *et al.* (*loc. cit.*) reported the results of fractionation of serum protein in 10 cases of lymphogranuloma venereum. They found that hyperglobulinæmia in venereal lymphogranuloma was associated with an elevation of *euglobulin* and *pseudoglobulin I.* Their spread of the values in g. per 100 c.c. were : for total globulin, 7·7 g. to 3·5 g. ; for *euglobulin*, 3·4 g. to 0·5 g. ; for total *pseudoglobulin*. 4·3 g. to 2·4 g.

The results of fractionation of plasma proteins by the method of Howe are recorded in 30 cases of syphilis and 12 cases of venereal lymphogranuloma. Hyperproteinæmia was present in 9 of the 12 cases of lymphogranuloma. Twelve patients with syphilis presented protein level above 8 g. per 100 c.c., 18 did not have associated hyperproteinæmia. Unfortunately Frei test was not done in any of our cases.

The results of fractionation of plasma protein in 30 patients with syphilis are presented in Table III. Globulin subfractions were not determined in 8 of these cases.

TABLE III.

Plasma protein values in syphilis.

Number.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.
	Results expressed in g. per 100 c.c.					
	A.—Cases with hyperproteinæmia.					
1	8·75	4·82	3·93	1·32	2·07	0·54
2	8·75	4·62	4·13	1·48	2·27	0·35
3	8·62	4·41	4·21	0·82	2·77	0·62
4	8·37	4·19	4·18	1·15	2·16	0·87
5	8·37	3·65	4·72	1·60	2·87	0·25
6	8·31	3·78	4·53	1·19	3·03	0·31
7	8·25	4·26	3·99	1·86	1·87	0·26
8	8·21	4·22	3·99	1·27	2·34	0·38
9	8·18	4·75	3·43	1·08	1·90	0·45
10	8·12	4·00	4·12	1·42	2·42	0·28
11	8·00	4·94	3·06	0·72	2·34	Serum.
12	8·00	4·71	3·29	1·15	2·14	,,

TABLE III—*concld.*

Number.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.
			B.—Normal total protein level with hyperglobulinæmia.			
13	7·87	4·81	3·06	0·60	2·17	0·29
14	7·81	4·52	3·29	1·08	2·21·	Serum.
15	7·79	3·49	4·30	1·05	2·45	0·80
16	7·70	3·20	4·50	1·40	2·65	0·45
17	7·68	4·12	3·56	0·79	2·46	0·31
18	7·68	4·22	3·46	1·03	2·17	0·26
19	7·50	3·93	3·57	0·67	2·70	0·20
20	7·31	3·37	3·94	0·95	2·74	0·25
21	7·18	4·18	3·00	0·83	2·17	Serum.
			C.—Globulin subfraction not determined.			
22	7·87	3·93	3·94	...	...	...
23	7·86	3·30	4·56	...	...	...
24	7·62	3·82	3·80	...	...	...
25	7·60	3·47	4·13	...	...	...
26	7·31	4·00	3·31	...	...	...
27	7·31	3·86	3·45	...	...	...
28	7·23	3·91	3·32	...	...	...
29	6·95	3·40	3·55	...	...	...
30	6·85	3·35	3·50	...	...	...

From the results of Table III it will be seen that in syphilis the globulin fraction is definitely increased. The concentration of total globulin was above 3 g. per 100 c.c. in all the cases and above 4 g. in 10 of the 30 cases studied. The spread of total globulin is from 3·0 g. to 4·72 g. per 100 c.c., with more than three-quarters of the results falling between 3·4 g. and 4·5 g. There is no significant difference in the value of globulin between those presenting hyperproteinæmia and those in which the total protein is within normal range. The hyperglobulinæmia in syphilis is due to an elevation of both euglobulin and pseudoglobulin. The values of fibrinogen was decidedly in the upper range of normal in most of the cases and elevated in of the 30 cases studied.

Hyperglobulinæmia in syphilis is not associated with any marked lowering of albumin as is seen in most of the chronic infections. The value of albumin above 4 g. per 100 c.c. was found in 16 of the 30 cases and values between 3·2 g. and 4·0 g. were found in 14 others. The range of values of albumin under A—3·65 g. to 4·82 g. per 100 c.c.—as well as their distribution are at a somewhat higher level than the corresponding values under B. The value of total protein in series A is, therefore, higher than those in series B.

TABLE IV.

Fractionation of plasma proteins in venereal lymphogranuloma.

Number.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.
			Results expressed in g. per 100 c.c.			
1	9·82	3·81	6·01	2·56	2·83	0·62
2	9·81	3·66	6·15	3·21	2·44	0·50
3	9·68	3·00	6·68	3·06	2·92	0·70
4	9·56	3·71	5·85	1·91	3·37	0·57
5	9·50	3·91	5·59	2·43	2·88	0·28
6	9·50	3·63	5·87	2·56	2·39	0·92
7	8·25	3·38	4·87	1·90	2·71	0·26
8	8·25	3·13	5·12	2·00	2·17	0·95
9	8·06	2·81	5·25	3·11	1·52	0·62
10	8·00	3·63	4·37	1·96	2·01	0·40
11	7·58	3·38	4·20	1·86	1·92	0·42
12	7·62	2·12	5·50	3·56	1·99	Serum.
13*	7·18	4·17	3·01	0·84	2·17	,,
14	9·56	3·63	5·93	3·81	2·12	,,
15	9·06	3·19	5·87	3·58	2·29	,,

*A case of suspected lymphogranuloma venereum.

Of the 14 patients with venereal lymphogranuloma 12 presented values of total protein above 8 g. per 100 c.c., and 2 did not have associated hyperproteinæmia. The spread of total protein is from 7·58 g. to 9·82 g. per 100 c.c. This range as well as their distribution is in agreement with the results recorded by Gutman et al. (loc. cit.) in their 9 cases of lymphogranuloma venereum. The value of 11·2 g. encountered in case 1 of their series is considerably higher than the value of 9·82 g. per 100 c.c. which is highest in our series.

The range of albumin varying from 2·81 g. to 3·91 g. per 100 c.c. compares well with the range of 2·7 g. to 3·7 g. reported by those authors except for the high value of 4·7 g. per 100 c.c. obtained in case 7 of their series.

The concentration of globulin above 5 g. per 100 c.c. was observed in 9 of the 12 cases. The value of 6·68 g. which is highest in our series is considerably lower than the value of 7·7 g. per 100 c.c. reported by Gutman et al. (loc. cit.). The range as well as the distribution of the values of globulin are essentially the same as reported by those workers. The globulin increment in venereal lymphogranuloma is composed largely of euglobulin fraction of the total globulin. The average value of euglobulin in this series is about four times the normal range, the highest value being 3·21 g. per 100 c.c. The concentration of pseudoglobulin was above the normal range in all but 1 case. The fibrinogen is considerably raised in most of the patients with venereal lymphogranuloma.

Howe's protein fractions in venereal lymphogranuloma appears to be significantly different from those in syphilis. Analysis of the available data reveals that the total globulin is considerably raised in all patients with lymphogranuloma. The average value of globulin in this series is 5·4 g. per 100 c.c. At such a high globulin level, the globulin increment is composed largely of euglobulin fraction which in 5 of the 12 cases superseded total pseudoglobulin as the major globulin component. The euglobulin content varied from 32 to 56 per cent of the total globulin in this series. In contrast to this, the mean value of globulin in patients with syphilis is only 3·8 g. per 100 c.c. and the mean euglobulin content being 1·1 g. per 100 c.c. or 29 per cent of the total globulin. This appears to be a significant difference and points to lymphogranuloma rather than to syphilis as the principal cause of hyperglobulinæmia with associated hyperproteinæmia.

The blood from case 13 (Table IV) was sent for the determination of plasma protein and was referred by the clinician as a case of suspected lymphogranuloma venereum. An analysis of the result offered evidence to the contrary; subsequently, the case was clinically declared as one of tubercular adenitis.

Fractionation of plasma proteins was carried out in 3 cases of tubercular lymphadenitis for the sake of comparison. The results are recorded in Table V.

All the 3 patients were females and reported for swelling of lymph glands in the region of neck or axilla. All the patients complained of fever off and on. The duration of illness was 1, 3 and 5 years respectively. The general nutrition of the patients appeared good. On screening no lesions were detected in the lungs. Clinical examination showed signs of tubercular adenitis. The findings were confirmed by operation and histological examination.

TABLE V.

Fractionation of plasma protein in tubercular adenitis.

Case number.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.
			Results expressed in g. per 100 c.c.			
1	8·62	5·30	3·32	1·20	1·90	0·22
2	8·26	4·47	3·79	1·12	2·42	0·25
3	8·62	4·21	4·41	1·41	2·66	0·31
3*	8·37	4·22	4·15	1·56	2·59	Serum.

*14 days after the removal of the gland.

From the results of Table V it is evident that the results of fractionation of plasma protein in tubercular adenitis is significantly different from those of lymphogranuloma venereum. The range of values of albumin in these 3 patients was higher than those observed in lymphogranuloma. The significant difference between the two groups was in the value of globulin which was considerably higher in lymphogranuloma venereum than in tubercular adenitis. The increase in globulin was due to a greater increase in the euglobulin fraction. The values of pseudoglobulin in both the groups were very nearly the same. That the removal of the glands made no difference in the values of different protein fractions is clearly shown by the result of case 3.

FRACTIONATION OF PLASMA PROTEIN IN TUBERCULOSIS.

Eichelberger and McCluskey (1927) reported the results of 150 determinations of plasma protein on their 109 patients with tuberculosis. The total protein was above 8 g. per 100 c.c. in 41 per cent of the determinations, the globulin above 3 g. in 62 per cent and albumin below 4 g. in 17 per cent. In none of their patients the total protein concentration was below normal. Kagan (*loc. cit.*) presented a summary of the results of total protein and of globulin in 12 patients with tuberculosis. A study of his table reveals that in 4 of the 29 determinations the total protein was above 7·5 g. per 100 c.c. and the value of globulin was above 3 g. per 100 c.c. in 16 of the 20 determinations.

The present series includes the results of fractionation of plasma proteins in 35 patients with tuberculosis at the time of admission to the hospital. Only 7 patients presented values of total protein above 8 g. per 100 c.c. In 1 patient with pleurisy with effusion the total protein was 8·75 g. per 100 c.c. The results are given in Table VI.

TABLE VI.

Fractionation of plasma proteins in tuberculosis.

Number.	Total. protein.	Albumin.	Globulin.	Euglobulin.	Pseudoglobulin.	Fibrinogen.	DIAGNOSIS.
	Results expressed in g. per 100 c.c.						
	A.—Cases with hyperproteinæmia.						
1	8·93	3·47	5·46	1·99	2·70	0·77	Bilateral pulmonary T. B.
2	8·75	3·05	5·70	2·50	2·90	0·30	Bilateral tuberculosis.
3	8·50	4·44	4·06	0·94	2·16	0·96	Suspected T. B. Two sisters died of tuberculosis.
4	8·37	3·47	4·90	1·10	2·53	1·27	Tuberculosis of right lung with cavity, sputum + for tuberculosis.
5	8·28	3·68	4·60	1·48	3·12	Serum	Tuberculosis and· cirrhosis.
6	8·25	4·47	3·78	1·10	2·49	0·19	Suspected pulmonary T. B.
7	8·06	3·34	4·72	1·81	2·42	0·49	...
8	8·00	3·33	4·67	1·09	2·40	1·18	Extensive Bilateral T. B.
	B.—Normal total protein with hyperglobulinæmia.						
9	7·90	3·47	4·43	1·51	2·44	0·48	Pulmonary tuberculosis.
10	7·68	4·17	3·51	0·59	2·62	0·30	Hæmoptyses once, involvement of right lung.
11	7·68	3·71	3·97	1·17	2·09	0·71	Tuberculosis of right lung.
12	7·66	2·86	4·80	1·76	2·71	0·33	Streaks of infiltration both upper zone. Sputum + for tuberculosis.
13	7·62	3·82	3·80	0·64	2·92	0·24	Bilateral tuberculosis.
14	7·56	3·56	4·00	1·35	1·90	0·75	Tuberculosis of right lung with cavity.
15	7·50	2·99	4·51	0·85	2·76	0·90	T. B. of the right lung with cavity.
16	7·37	3·84	3·53	0·68	2·27	0·58	Pulmonary tuberculosis.
17	7·37	2·96	4·41	1·22	2·49	0·70	Extensive bilateral T. B.
18	7·13	3·66	3·47	0·69	2·34	0·44	Involvement of right lung. Sputum + for tuberculosis.
19	7·00	3·50	3·50	0·53	2·01	0·96	Involvement of the extensive right side. Sputum + for T. B.

TABLE VI—*concld.*

Number.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudoglobulin.	Fibrinogen.	DIAGNOSIS.
C.—Total protein below normal with hyperglobulinæmia.							
20	6·83	2·27	4·56	1·50	2·31	0·75	Diffuse infiltration both lungs.
21	6·83	1·54	5·29	1·40	3·14	0·75	Tuberculosis of right lung with cavity.
22	6·60	3·60	3·00	0·84	2·16	Serum	Bilateral tuberculosis.
23	6·31	2·90	3·41	0·65	2·21	0·55	„ „
24	6·06	1·86	4·20	0·83	2·47	0·90	Bilateral tuberculosis with cavity.
25	5·75	2·59	3·16	0·70	2·00	0·46	Infiltration right side with adhesion mid-zone.
26	5·68	2·96	2·72	0·70	1·61	0·41	Hæmoptyses once. Extensive infiltration of right lung.
27	5·12	3·00	2·12	0·75	1·37	Serum	Hæmoptyses, loose stool, extensive right side with cavity.
D.—Globulin subfraction not determined.							
28	7·90	4·00	3·90	...	...	...	Hæmoptyses. Bilateral tuberculosis.
29	7·60	4·59	3·01	...	...	...	Admitted for hæmoptyses, bilateral tuberculosis, sputum + for T. B.
30	7·50	2·09	5·41	...	...	...	Tuberculosis of right lung upper lobe with cavity.
31	6·93	2·87	4·06	...	...	...	Tuberculosis of right lung with cavity.
32	6·81	2·65	4·16	...	...	...	Tuberculosis of right apex. Patient recovered.
33	6·81	3·44	3·37	...	...	...	Hæmoptyses twice. Sputum + tuberculosis left lung with cavity.
34	6·75	2·72	4·03	...	...	...	Involvement of right side, sputum +.
35	6·90	2·74	3·16	...	...	...	Extensive tubercular infiltration of left side with dense pleural thickness.
36	5·03	2·53	2·50	...	...	...	Extensive bilateral, sputum +.

From the results of Table VI it can be seen that the value of total protein was normal in 15 and below normal in 13 others. In only 7 of the 35 cases was the total protein above 8 g. per 100 c.c.

The total globulin was above normal in all but 2 cases. In only 4 of the 35 cases the concentration of globulin exceeded 5 g. per 100 c.c. From the trend of the values it would appear that the usual range of globulin in tuberculosis is from 3·5 g. to 5 g. per 100 c.c. Hyperglobulinæmia in tuberculosis was associated with a consistent increase in the pseudoglobulin fraction. The euglobulin showed a wide range of variation from a normal value of 0·35 g. to a value as high as 2·5 g. per 100 c.c. A marked increase in the value of fibrinogen appears to be an important feature of the disease.

Hypoalbuminæmia was seen with greater frequency in this series. The concentration of albumin was below 3 g. in 14 cases and varied between 3 g. and 4 g. per 100 c.c. in 15 others. In only 5 of the 35 cases the value of albumin was above 4 g. per 100 c.c. Malnutrition was probably responsible for the low value of albumin observed in many of our patients.

Boissevain (1940) reported that serum albumin promotes the growth and serum globulin inhibits the growth of the tubercle bacilli in artificial medium. Luetscher (1941) from the study of blood proteins by electrophoretic method in 2 cases of tuberculosis reported that the findings in one was essentially normal and in the other γ globulin was elevated 32 per cent, while the α and β globulin was normal. Seibert and Nelson (1942) reported moderate increase of γ globulin at the expense of albumin in 3 cases of advanced tuberculosis. The same authors reported that in experimental tuberculosis in rabbits there is at first an increase in α and β globulin, and then an increase in γ globulin which they felt accompanied resistance to disease. In the light of these observations it seems not very unlikely that the consistent increase in the pseudoglobulin fraction observed in this series is partly due to an increase in the γ globulin. The presumptive assumption of the association of γ globulin with the pseudoglobulin fraction might be clarified by comparing the electrophoretic pattern with the Howe's fractions.

Plasma Proteins in Patients with Tuberculosis Declared Clinically Inactive.

It is of interest to note that normal values of plasma proteins were found in 4 patients with tuberculosis in whom all clinical signs of activity disappeared. The results are given in Table VII.

The results in Table VII reveal the normal values of different protein fractions in all these patients after their recovery from tuberculosis. A progressive rise in the value of albumin to normal level and a gradual fall of globulin due to a lowering of euglobulin are the significant alterations of plasma proteins that accompany the process of recovery. The lower range of pseudoglobulin in all these cases may be indicative of the absence of sufficient antibody formation. The prognosis as to the complete recovery is, however, difficult to determine. Howe's method of fractionation of plasma proteins, despite its deficiencies as compared with the electrophoretic method, give fairly uniform results and may prove to be of great prognostic value in following the course of the disease.

TABLE VII.

Howe's protein fractions in patients with tuberculosis declared clinically inactive.

Number.	Date.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.
		Results expressed in g. per 100 c.c.					
1	19–12–41*	6·81	2·65	4·16	...	...	...
	20– 5–44	7·57	4·77	2·80	1·18	1·62	Serum.
2	1944	6·90	4·20	2·70	0·95	1·54	0·21
	1946	7·40	4·89	2·61	0·68	1·62	0·21
3	1942	6·93	4·37	2·56	0·62	1·94	Serum.
4	1943	7·67	4·97	2·70	0·58	1·95	0·17
	1945*	7·67	4·22	3·45	1·61	1·50	0·34
	Jany. 1946*	7·50	4·13	3·37	1·37	1·62	0·38
	June 1946	7·37	4·62	2·75	0·68	1·75	0·35
5	...	7·60	5·18	2·42	0·60	1·62	0·20

* During illness.

CHANGES IN THE PLASMA PROTEINS IN KALA-AZAR.

The results of fractionation of plasma proteins in 2 patients with kala-azar during treatment are shown in Table VIII.

TABLE VIII.

Fractionation of plasma proteins in 2 cases of kala-azar during the course of treatment.

Date.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.

Results expressed in g. per 100 c.c.

Date.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.
Case 1.—						
21–7–42	11·43	2·37	9·06	4·02	4·38	0·66
10–8–42	11·64	3·04	8·60	3·80	4·25	0·55
21–8–42	11·62	3·52	8·10	3·49	4·10	0·51
31–8–42	10·47	3·91	6·56	2·16	3·80	0·60
7–9–42	10·53	4·29	6·24	2·28	3·36	0·60
15–9–42	9·87	4·38	5·49	2·03	2·87	0·59
21–9–42	9·85	4·44	5·41	1·36	3·35	0·70
Case 2.—						
28–12–42	9·06	1·50	7·56	4·63	2·56	0·37*
4– 1–43	9·18	1·07	8·11	4·80	3·11	0·20†
15– 1–43	9·37	1·28	8·09	4·79	3·03	0·27
6– 2–43	9·72	1·90	7·82	4·72	2·86	0·24
16– 2–43	9·87	2·39	7·48	4·65	2·57	0·26
5– 3–43	10·43	3·25	7·18	3·02	2·87	0·29

* Treated with quinine.   † Treatment changed to urea stibamine.

A study of the results of Table VIII clearly indicate that the values of plasma proteins were typical of kala-azar characterized by a low albumin and a marked

increase in the value of globulin. The globulin increase was due to a greater increase in the euglobulin than in the pseudoglobulin fraction. The concentration of euglobulin was about 7 times the normal value. Hyperglobulinæmia in both the cases was associated with hyperproteinæmia. The prompt response to treatment shown by case 1 was reflected by a progressive rise in the value of albumin to normal level and a gradual fall in the value of euglobulin. The concentration of globulin remained elevated in spite of a regular fall in the value of euglobulin. The partial recovery of case 2 was followed by an increase in the value of albumin which was slow but unmistakable. The globulin value differed little from the highest value reached during the illness. Although there is a definite tendency for the globulin fraction to return to normal level little is known of the duration of hyperglobulinæmia.

From the results of these cases of kala-azar it is evident that serial observations are of great value, first because these give information concerning the efficacy of treatment ; second because these are an index of the progress of the disease.' The progressive rise of albumin towards normal is of definite prognostic value in following the course of the disease.

Lloyd and Paul (*loc. cit.*) followed the changes in the plasma proteins in 6 cases of kala-azar by the refractrometric method. According to these authors the first stage in the treatment is a fall in the pseudoglobulin fraction and a rise of albumin which occurs immediately. The albumin and the total globulin curves rapidly crossing to form a characteristic × curve. In the second stage, the pseudoglobulin after an initial fall begins to rise and steadily climbs up, the euglobulin falling by an equal gradient. The change of euglobulin to pseudoglobulin was regarded by them as the explanation of the second stage and the conversion of pseudoglobulin to albumin as the explanation of the first stage.

We are, however, led to believe that the rapid rise in albumin immediately after the administration of antimony would tend to verify the importance of the infectious process *per se* in interfering with the production of albumin. The progressive rise in albumin during the treatment could be better attributed to the regeneration of albumin by the liver after the termination of the infection rather than to the transformation of pseudoglobulin to albumin as suggested by Lloyd and Paul (*loc. cit.*). It is evident from the results of our 2 cases of kala-azar that the increase in the concentration of albumin is decidedly greater than the decrease in pseudoglobulin at any stage during the course of clinical improvement. Besides, Hartley (1915) has shown that albumin and globulin differ decidedly in their amino-acid composition, consequently hypothesis assuming the transformation of globulin to albumin or vice versa must receive more definite proof in order that they may deserve further consideration.

The intimate association of liver with the maintenance of normal concentration of proteins of the blood plasma is well established. Although the cases of kala-azar are accompanied by hepatic involvement, the ability of the liver to form plasma albumin is, however, not impaired. This is clearly indicated by the rapid rise in albumin which follows immediately after the administration of antimony. Presumably this means that the normal plasma-protein forming mechanism is disturbed by the complicated body reaction to infection.

There is now good evidence that production of serum globulin is dependent upon the utilization of an adequate assortment of amino-acids. Although complete amino-acid analysis of normal serum globulin is still lacking, there is evidence that it contains several amino-acids essential for maintenance of nitrogen equilibrium in man. Madden and Whipple (1940) have shown 'that globulin formation is directly dependent upon diet'. In kala-azar, when the parasites invade the organism, the essential amino-acids derived from the food are mostly utilized for the formation of globulin which takes place at the expense of albumin. The protein reserve of the body becomes dissipated in an attempt to maintain the normal level of plasma albumin and as the compensatory mechanism fails to make good the loss, the albumin gradually decreases until the vicious circle is broken by antimony. When, therefore, the source of infection is removed, the essential amino-acids become available for the synthesis of albumin resulting in a rapid rise of albumin. A decrease in globulin formation that accompanies the rise of albumin might be due to lessened stimulus to globulin formation resulting from the action of the drug on the invading organism. The significance of increased globulin in kala-azar is not properly understood.

Hyperglobulinæmia and Associated Hyperproteinæmia in some types of Hepatic Disease, especially Cirrhosis.

An alteration in serum proteins during the course of cirrhosis and other types of liver disease has been observed by several investigators. Myers and Keefer (1935) and Tumen and Bockus (1937) reported that hypoalbuminæmia was the most constant alteration of serum protein in all patients who had chronic or advanced diseases of the liver. An elevation in the serum globulin was not as constant as the reduction of serum albumin. Post and Patek (1942) reported that a reduction of serum albumin is an essential factor in the production of ascites and that the prognosis as to the duration of life becomes increasingly grave as the level of serum albumin decreases in patients with cirrhosis.

Recently, various authors have demonstrated a rise in serum globulin in some cases of cirrhosis of liver. Gutman and Wise (1936) reported globulin values of 6·0 g., 5·6 g. and 5·0 g. per 100 c.c. with corresponding total protein values of 8·4 g., 7·4 g. and 7·2 g. in 3 cases of cirrhosis. Cardon *et al.* (*loc. cit.*) reported 9 cases of hepatic diseases including 6 cases of cirrhosis in which hyperglobulinæmia was associated with hyperproteinæmia. Stacey (1945) reported the results of fractionation of serum proteins in 36 cases of portal cirrhosis. The value of total protein above 8 g. per 100 c.c. was found in 3 of the 36 cases studied.

The data contained in this report represent part of the results of fractionation of plasma proteins in 56 patients with cirrhosis of liver. Ten patients presented a value of total protein above 8 g. per 100 c.c. This series consisted of 16 cases in adults and 40 others in children including some cases of infantile cirrhosis.

The results of fractionation of proteins in the blood plasma of 16 patients in adults and those of 6 patients in children presenting hyperproteinæmia are recorded in Table IX. These results indicate the general trend of values of plasma protein in cirrhosis.

TABLE IX.

Fractionation of plasma proteins in cirrhosis of liver.

Number.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.	Date.
	Results expressed in g. per 100 c.c.						
	Group A.—6 cases in children presenting hyperproteinæmia.						
1	8·12	1·69	3·43	1·50	1·93	Serum.	...
2	8·31	4·16	4·15	1·64	2·30	0·21	...
3	9·62	3·89	5·73	3·10	2·31	0·32	...
4	9·30	3·74	5·56	2·87	2·34	0·35	...
5	8·00	2·63	5·37	2·78	2·28	0·31	...
6	8·10	1·79	6·31	3·69	2·39	0·23	...
	Group B.—15 cases in adults.						
1	10·87	2·56	8·31	5·16	2·98	0·17	18–10–44
	10·81	2·81	8·00	5·61	2·22	0·17	24–10–44
2	9·61	2·55	7·06	4·89	2·00	0·17	...
3	8·70	2·57	6·13	2·88	2·98	0·27	5– 2–44
	9·06	2·86	6·20	2·70	3·25	0·25	20– 2–41
4	7·81	2·32	5·49	3·12	2·22	0·15	...
5	7·81	2·81	5·00	2·99	1·65	0·36	...
6	7·37	2·94	4·43	1·41	2·76	0·26	...
7	6·88	2·12	4·76	1·67	3·09	Serum.	...
8	6·68	2·57	4·11	1·48	2·44	0·19	...
9	6·56	2·29	4·27	2·34	1·71	0·22	...
10	6·43	2·27	4·16	1·25	2·41	0·50	...
11	6·37	2·45	3·92	2·17	1·51	0·24	...
12	6·31	2·56	3·75	1·62	1·94	0·19	...
13	6·00	2·41	3·56	1·37	1·90	0·29	...
14	5·81	1·98	3·83	1·69	1·82	0·32	...
15	5·25	2·68	2·57	1·05	1·32	0·20	...
16	8·54	4·22	4·32	1·49	2·17	0·66	...

All of the 15 cases included under group B were males and attended the hospital when the disease was well advanced. All of them had ascites. In every instance the plasma albumin was below 3 g. per 100 c.c. The globulin was above the normal range in all but one. The globulin level was more variable than that of the albumin. The value of 8·31 g. per 100 c.c. recorded in case 1 is possibly the highest value on record in this condition. The total protein was normal in 3, below normal in 9 cases. Hyperglobulinæmia was associated with hyperproteinæmia in 3 instances.

In 22 of the 40 cases in children the values of plasma proteins were similar to those found in adults. The albumin varied between 1·4 g. and 2·84 g. per 100 c.c. The globulin was elevated in 17 of the 22 cases ranging between 2·56 g. and 6·08 g. per 100 c.c. The total protein was above 8 g. per 100 c.c. in only 2 cases (cases 5 and 6, group A).

In contrast to the foregoing data are those of 14 patients in children who appeared to be less severely ill. The range of albumin in this group was higher, varying from 3·13 g. to 3·80 g. per 100 c.c. The globulin values showed less marked increase ranging between 3·06 g. and 4·06 g. per 100 c.c. The total protein varied between 6·56 g. and 7·62 g. per 100 c.c. These patients were admitted for irregular fever with distension of abdomen and jaundice in some of the cases. Nevertheless, all of them revealed ample signs of cirrhosis of liver.

The results of the remaining 4 children presenting hyperproteinæmia are presented in group A (cases 1 to 4). The results of these 4 cases are significantly different from those of the other cases in the series in that the hyperglobulinæmia in these patients was not accompanied by an appreciable lowering of albumin which is so common in this condition.

The most significant alterations of plasma proteins in cirrhosis of liver are a decrease in the value of albumin and an increase in globulin. The incidence of marked hypoalbuminæmia seems to be usually high. Thirty-nine of the 56 cases presented albumin levels below 3 g. per 100 c.c. Our figures with regard to the low albumin in cirrhosis play only a confirmatory rôle; an increase in the value of globulin was, however, constant in this series as the lowering of albumin. The total globulin above normal was found in 50 of the 56 cases studied. The data presented in Table IX reveal that the values of globulin show a wide range of variation without a significant change in the concentration of albumin.

Our findings when taken as a whole do not justify any definite conclusion as to the correlation between the changes in the albumin and globulin levels and the clinical course of the disease. Gray (1940) suggested that there may be a correlation between the extent of alteration of albumin : globulin ratios and the severity of hepatic disease. Post *et al.* (*loc. cit.*) reported that there is a direct correlation between the level of serum albumin and the clinical course. From the trend of the changes in the plasma proteins it would appear that the albumin content of the blood tends to decrease as the disease process continues until it reaches a low level. But the significance of a wide range of variation in the value

of globulin without any appreciable change in the concentration of albumin cannot, however, be overlooked. For the interpretation of results it is essential that other factors which may affect the concentration of either of the constituents and account for lack of correlation must be excluded. The co-existence of nutritional deficiency and cirrhosis of the liver may be responsible for the low level of albumin in many of our cases. No satisfactory explanation can, however, be advanced for the occurrence of marked hyperglobulinæmia and hyperproteinæmia in some cases of cirrhosis and not in others.

The interpretation of the different globulin subfractions in hyperglobulinæmia are more obscure. Gutman *et al.* (*loc. cit.*) was the first to notice an increase of euglobulin in hepatic cirrhosis. Stacey (*loc. cit.*) reported that serum euglobulin was above normal in all the patients with cirrhosis, the average being four times the normal average. The data recorded in Table IX indicate that an increase in the euglobulin fraction being the sole cause of hyperglobulinæmia in this series. The pseudoglobulin fraction was slightly above normal range in 11 of the 22 cases, but the average show a small rise which is statistically significant. An inspection of our data further reveals that as the total globulin content increases beyond 5 g. per 100 c.c., the globulin increment is composed almost entirely of euglobulin fraction which in 10 of the 22 cases has superseded the total pseudo-globulin as the major globulin component. The highest value of euglobulin in this series is 5·61 g. per 100 c.c. which is about ten times the normal average. Should such marked increase in euglobulin fraction be found to be of such high incidence and so characteristic of hepatic cirrhosis it should have a definite clinical significance. Stacey has suggested that estimation of serum euglobulin and euglobulin : albumin ratio may provide more sensitive indices of disordered protein formation in this disease than total globulin. It is evident that further investigation is necessary in order to explain fully the mechanism of euglobulin formation in hepatic disorders and also to search for some common factor responsible for the increase in euglobulin observed in a number of other conditions which are clinically so different.

PLASMA PROTEIN IN HEPATIC DISORDERS OTHER THAN CIRRHOSIS.

Determination of plasma protein was carried out on more than 30 patients with hepatic involvement of various kinds such as toxic hepatitis, obstructive jaundice, liver abscess and so-called catarrhal jaundice. Elevation of globulin was noted in all the cases but not with the same degree as in the cirrhosis. The globulin values ranging between 2·87 g. and 4·37 g. per 100 c.c. and exceeding the value of 4·0 g. in 6 of the 32 cases studied. The values of albumin were within the normal range in 8 cases, below 4 g. per 100 c.c. in 14 cases and varying between 2 g. to 3 g. per 100 c.c. in 10 others. The values of total protein ranging between 6 g. to 7·93 g. per 100 c.c. in 30 of the 32 cases. Hyperproteinæmia was present in 2 cases of acute catarrhal jaundice. In one of the patients the protein fractions returned almost to normal within 15 days after clinical improvement. The results are recorded in Table X. This patient was a member of the staff whose blood was analysed 2 years previous to his illness and the result was included in the normal values of plasma protein in Indians.

TABLE X.

Plasma protein values in a case of catarrhal jaundice during illness and after recovery.

Date.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudo-globulin.	Fibrinogen.	Sedimentation rate.
			Results expressed in g. per 100 c.c.				
1941	7·96	5·21	2·71	0·65	1·71	0·35	3
1943*	8·90	5·01	3·89	1·52	2·13	0·24	34
1943†	7·93	5·08	2·85	0·54	2·12	0·22	3

* During illness. † 15 days after clinical improvement.

The changes in the plasma protein during an attack of acute catarrhal jaundice consisted in a decrease of albumin and an increase in the value of globulin. The globulin increment was found to be composed of both euglobulin and pseudoglobulin. Following recovery the globulin value almost returned to the original level. The decrease in the globulin level was entirely due to the lowering of euglobulin, the pseudoglobulin remaining unchanged. The result further indicates that an attack of catarrhal jaundice does not produce a significant decrease in the value of albumin.

HYPERPROTEINÆMIA IN MISCELLANEOUS CONDITIONS.

In the miscellaneous group in our series are 6 cases of acute nephritis, 5 cases of congestive heart failure, 5 cases of fever, 2 cases of suppurative infections and 1 in each of pneumonia, tetanus and primary toxic goitre. There was one case in which hyperproteinæmia was clearly due to dehydration from vomiting. The results are included in Table XI.

Acute nephritis.—Kagan (*loc. cit.*) reported that hyperglobulinæmia was seen in 50 per cent of the determinations done in a study of patients with acute glomerulonephritis. Hyperglobulinæmia was present in 80 per cent of the cases in our series. The concentration of globulin above 4 g. per 100 c.c. was found in only 13 of the 70 cases studied. There were only 4 cases in which the value of globulin exceeded 5 g. per 100 c.c. Hyperglobulinæmia associated with hyperproteinæmia was present in 6 of the 70 cases. The globulin increment in this condition was found to be composed of both euglobulin and pseudoglobulin, thus conforming with the characteristic pattern of chronic infection.

TABLE XI.

Hyperproteinæmia in miscellaneous conditions.

Number.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudoglobulin.	Fibrinogen.	DIAGNOSIS.
	Results expressed in g. per 100 c.c.						
1	9·97	5·34	4·63	1·91	2·12	0·60	Acute nephritis.
2	9·68	4·52	5·16	1·77	2·56	0·83	Hæmorrhagic nephritis.
3	8·87	3·69	5·18	2·00	2·23	0·95	Acute nephritis.
4	8·81	5·25	3·56	1·66	1·90	Serum	,, ,,
5	8·12	5·01	3·11	0·98	1·73	0·40	Nephritis.
6	8·08	3·48	4·60	1·34	3·26	Serum	,,
7	8·87	3·60	5·27	2·09	2·63	0·55	Congestive heart failure.
8	8·68	3·44	5·24	1·87	2·80	0·57	Cardiac failure.
9	8·31	4·66	3·65	1·10	2·19	0·36	,, ,,
10	8·06	3·40	4·66	1·30	3·11	0·25	,, ,,
11	8·07	3·37	4·70	1·90	2·80	Serum	Swelling of the feet and breathlessness.
12	9·87	4·44	5·43	1·77	2·34	1·32	Fits and fever.
13	9·37	3·00	6·37	3·74	2·37	0·26	Fever with enlarged spleen and liver.
14	8·93	4·45	4·48	1·69	1·92	0·87	During incubation period of malaria.
15	8·62	4·19	4·43	1·52	2·50	0·41	Typhoid, widal + 1 in 250.
16	8·19	3·51	4·68	1·73	2·95	Serum	Fever undiagnosed.
17	8·62	2·86	5·76	2·78	2·98	,,	Psoas abscess.
18	8·25	4·32	3·93	1·38	2·55	,,	Fever, swelling of the thigh-abscess.
19	8·31	3·54	4·77	2·54	2·05	0·18	Swelling of thyroid gland for 12 years.
20	8·18	3·50	4·68	2·02	2·66	Serum	Pneumonia, had gonorrhœa.
21	8·52	3·21	5·30	2·37	2·43	0·50	Tetanus.
22	9·00	3·32	5·68	2·45	3·23	Serum	Dehydration from vomiting, cause unknown.

TABLE XI—*concld.*

Number.	Total protein.	Albumin.	Globulin.	Euglobulin.	Pseudoglobulin.	Fibrinogen.	DIAGNOSIS.
			Normal total protein with marked hyperglobulinæmia.				
1	7·18	1·87	5·31	3·56	1·73	Serum	Kala-azar ; L.D. bodies present.
2	7·86	2·80	5·06	3·16	1·99	,,	Fever ; enlarged spleen and liver.

Congestive cardiac failure.—The concentration of albumin was found to be lowered in most of the cases of cardiac failure. In 70 per cent of the cases the values of albumin ranged between 3 g. and 4 g. per 100 c.c. The concentration of albumin below 3 g. per 100 c.c. was present in about 15 per cent of the cases. Our results with regard to the lowered range of albumin in congestive heart failure are in agreement with the findings of Payne and Peters (1932). The concentration of total protein was normal in 40 per cent of the cases and above 8 g. per 100 c.c. in 5 of the 50 cases studied. The normal value of total protein in spite of lowered albumin was due to an increase in the value of globulin. Globulin increase was present in 80 per cent of our cases of cardiac failure, a point which was noted by Luetscher (*loc. cit.*) in all their patients with congestive heart failure.

In addition to the cases already recorded under miscellaneous conditions there were a few more cases of hyperproteinæmia in this series. These cases have not been included under Table XI as the globulin subfractions was not determined in any of the cases.

Hyperproteinæmia was present in 5 of the 14 cases of tetanus studied. The tendency towards a higher range of total protein was noticed in almost all the cases due to a slightly elevated range of globulin. The concentration of globulin above 5 g. per 100 c.c. was found only in 1 case recorded in Table XI. Total protein above 8·7 g. and globulin above 5 g. per 100 c.c. were found in a patient who had pleurisy with effusion and also in 2 females who attended the hospital complaining of pain all over the joints. Hyperglobulinæmia was observed in a number of patients with various acute or chronic infections but the globulin increase was not significant.

DISCUSSION.

Total protein.—The highest value of total protein in our series was 11·62 g. per 100 c.c. and was found in a case of kala-azar. The concentration of total protein between 9 g. and 10 g. per 100 c.c. occurred mostly in lymphogranuloma venereum, in kala-azar and in cirrhosis. Less marked hyperproteinæmia was observed in a number of disorders associated with various infections.

Albumin.—The concentration of albumin within normal limits was found in all the 3 cases of tubercular adenitis and in some cases of syphilis. nephritis and a few other cases included under miscellaneous conditions. The values of albumin below normal were found in all conditions accompanying the wasting of chronic infections. In cirrhosis and in kala-azar the concentration of albumin was very often disproportionately low in relation to the total protein. The essential difference between the two conditions is that, when the patient with cirrhosis develops a definite hypoalbuminæmia, the chance of a subsequent sustained increase in this protein fraction is remote, whereas in a patient with kala-azar there is a progressive rise in the value of albumin immediately following treatment with antimony. There is no correlation, however. between the total protein and albumin in any of the conditions mentioned above. This is in agreement with the findings of Gutman *et al.* (*loc. cit.*).

Total globulin.—The highest value of total globulin in our series was 9·06 g. per 100 c.c. and occurred in a case of kala-azar. The values of total globulin varying between 6 g. and 8 g. per 100 c.c. were observed only in cirrhosis and values between 5 g. and 6 g. were most common in cases of lymphogranuloma venereum. The concentration of globulin was found to be elevated in almost all cases of syphilis. tuberculosis and nephritis. but only occasionally that the values exceeded 5 g. per 100 c.c.. the usual range being 3 g. to 4 g. per 100 c.c. An increase in the globulin fraction slightly above the normal range was encountered in persons apparently healthy but showing signs of mild or latent infection.

Graph 1 gives the relation of globulin to total protein and is made up of 12 points representing the results of 2 cases of kala-azar, 9 points for 9 cases of lymphogranuloma, 30 points for 30 cases of syphilis. 55 points for cirrhosis. 45 points for patients with tuberculosis, 4 points for each of the 4 cases of leukæmias and of tubercular lymphadenitis and 10 points for patients with various other infections. The points show a definite trend with a sharp positive slope indicating the direct proportionality of the total globulin to the total protein of the blood plasma.

Hyperproteinæmia is no doubt due to an increase in the value of globulin. yet a normal value of total protein may be obtained in spite of an increase in globulin due to a corresponding diminution in the albumin fraction as shown in Graph 1. Determination of total protein alone without reference to the separate components has no quantitative significance. It is for these patients that there is the greatest need for an accurate estimation of the albumin and globulin fractions separately.

Euglobulin and pseudoglobulin.—Several studies on serum protein by the method of Howe are recorded in the literature. The data relating to kala-azar. cirrhosis, lymphogranuloma venereum and various other infections indicate that hyperglobulinæmia is associated with an increase in euglobulin and pseudoglobulin fractions. The significance of the increase in any of the globulin subfractions is not properly understood.

From the results of fractionation of plasma protein. which we have carried out on a large series of cases covering wide varieties of diseases. it can be inferred that the range of variation of the two components of the globulin fraction may be

different in different infections. The values of euglobulin slightly above the normal range were found in individuals who were not actually ill when the blood was taken but showed signs of mild infections such as common cold or sore throat, etc. A similar increase in the euglobulin fraction was seen most frequently in all patients

GRAPH 1.
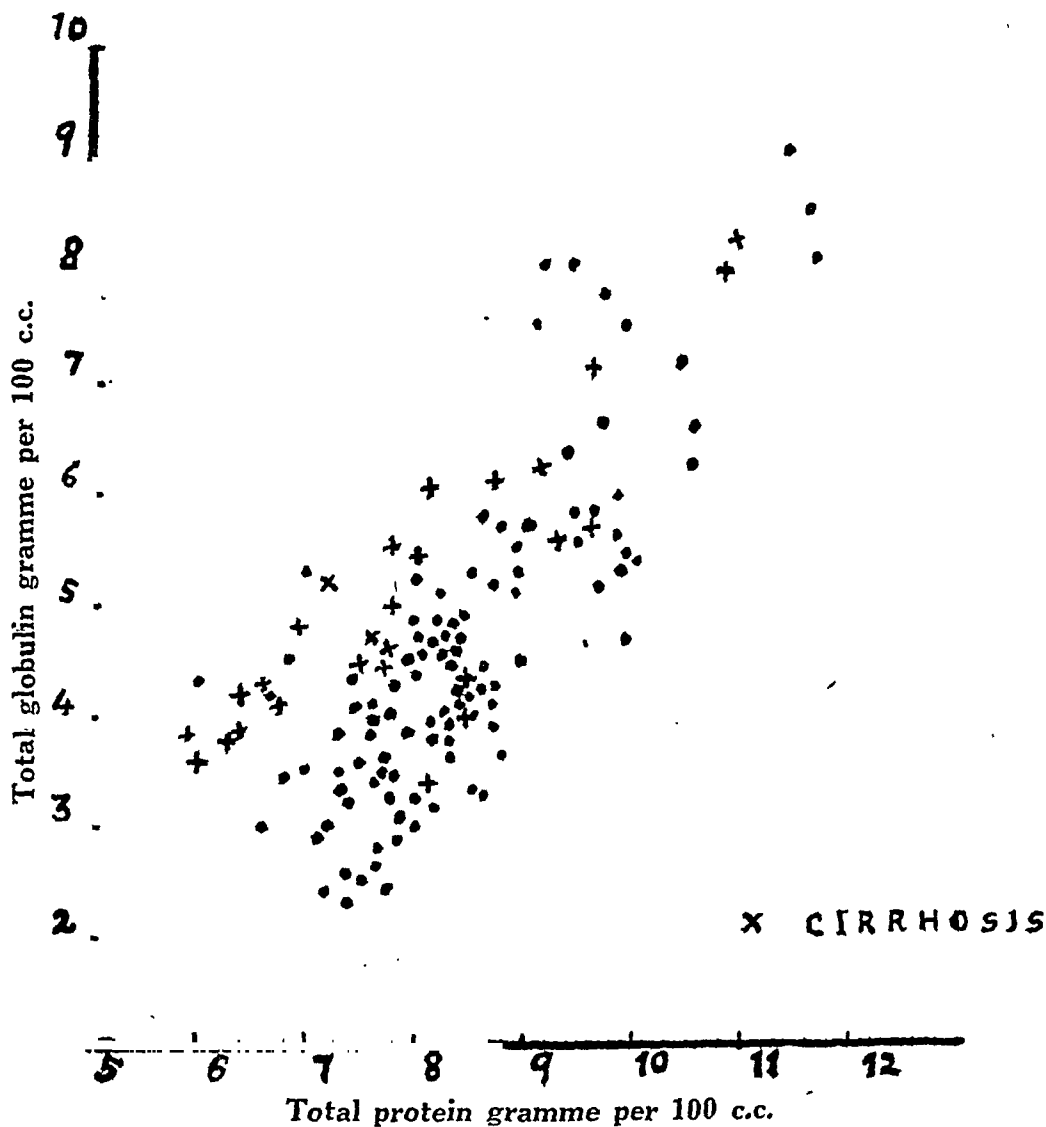
suffering from subacute nephritis or nephrosis. Patients presenting various manifestations of malnutrition, such as macrocytic anæmia or nutritional œdema, often showed values of euglobulin above normal range due to an associated infection. An increase in the concentration of euglobulin in all these cases was associated with a lowering of pseudoglobulin so that the total globulin was either normal

or slightly elevated. In contrast to these are the cases of acute nephritis, syphilis. tuberculosis, lymphadenitis, leukæmias, etc., where the increase in the euglobulin fraction was greater than those of the previous group and was associated with an increase in the pseudoglobulin. The total protein was, therefore. much above the normal.

A marked increase in the euglobulin fraction was encountered in almost all the cases of cirrhosis, lymphogranuloma and kala-azar. The increase in the euglobulin fraction was accompanied by a lesser increase in pseudoglobulin. The relation of euglobulin to the total globulin is shown in Graph 2. The points show

GRAPH 2.
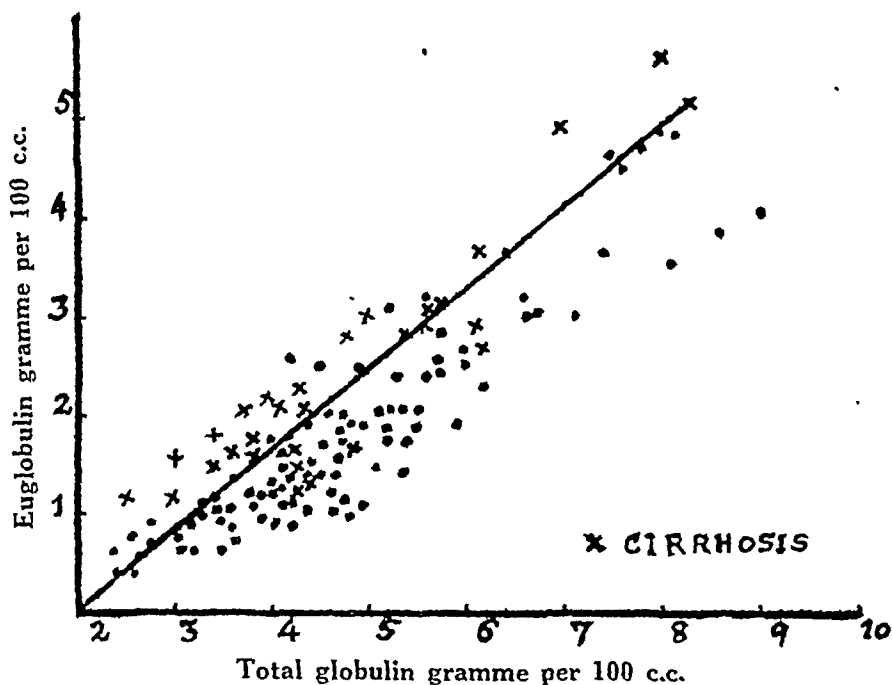
a definite upward trend illustrating that. as the concentration of globulin increases, a proportionately larger portion is composed of euglobulin.

A significant increase in the value of euglobulin is usually found to be associated with low albumin. Our results suggest that in some of the conditions, especially in kala-azar and in cirrhosis of the liver. the increase in the value of euglobulin takes place at the cost of albumin. so that. as the albumin : globulin ratio decreases, the euglobulin : albumin ratio increases.

By plotting the euglobulin : albumin ratio against the albumin : globulin ratio for various conditions mentioned in the text a curve is obtained as shown in

Graph 3. The points show that as the albumin : globulin ratio decreases, the euglobulin : albumin ratio show a slows but steady rise with a sharp upward trend

GRAPH 3.
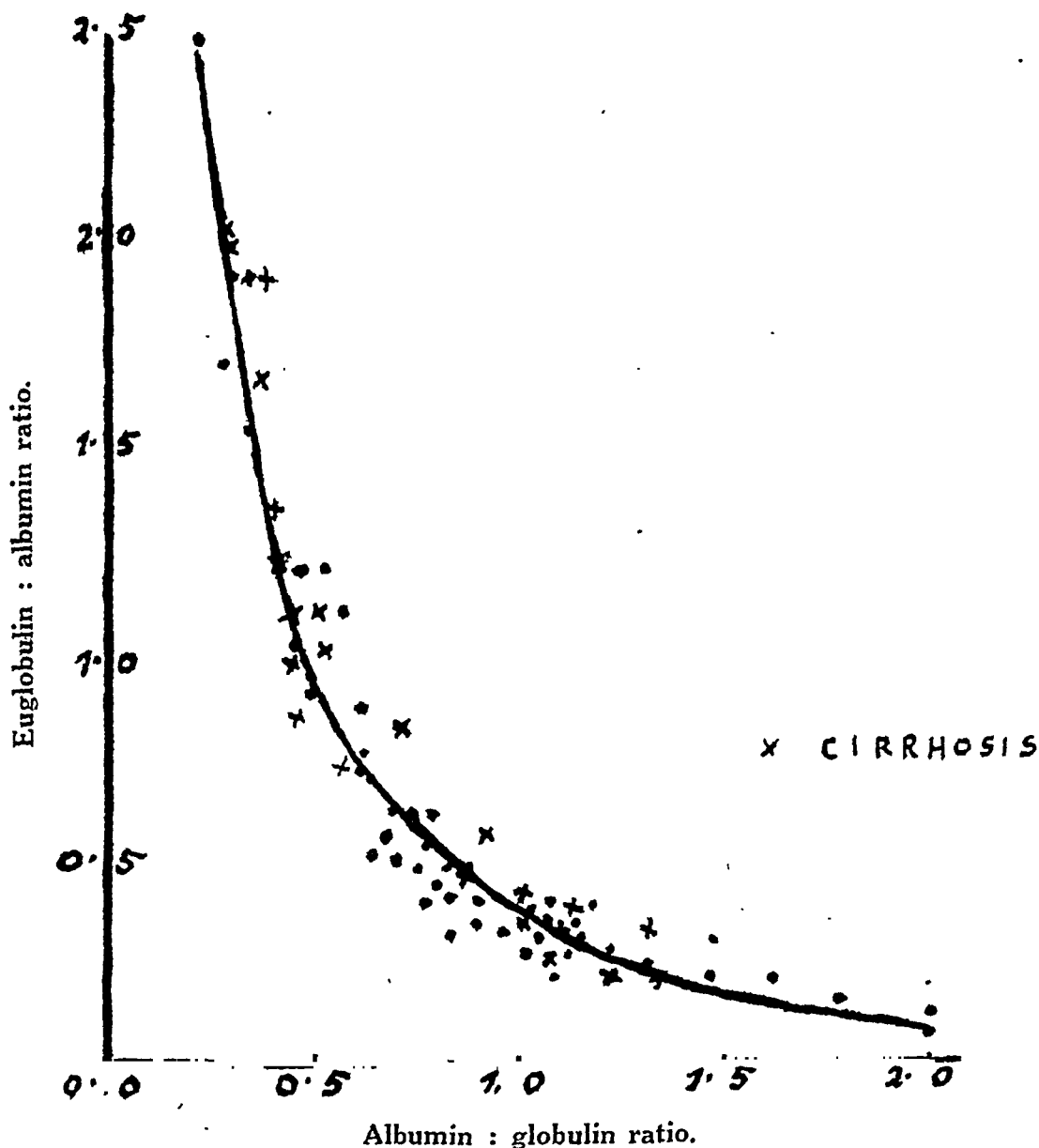
for values of albumin : globulin ratio below 0·5 ; illustrating that in some of the conditions the failure in the production of albumin is made good by increased euglobulin production.

Stacey (*loc. cit.*) suggested that euglobulin : albumin ratio may provide more sensitive indices of disordered protein formation in cirrhosis of the liver. This is in agreement with our findings. It is, however, necessary to exclude other conditions which may lead to a lowering of albumin. The significance of the increase in the value of euglobulin cannot be properly understood unless the common factor responsible for this increase in different conditions is found.

SUMMARY.

The results of fractionation of plasma proteins by the method of Howe are recorded in 74 cases of hyperglobulinæmia associated with hyperproteinæmia. The results illustrate the distribution of globulin subfractions in various disorders presenting hyperglobulinæmia. Hyperglobulinæmia is usually associated with an increase in both the euglobulin and pseudoglobulin fractions. In the higher range of globulin (above 5 g. per 100 c.c.) the globulin increment is mostly due to an increase in the euglobulin fraction. This is particularly significant in cirrhosis of the liver and in kala-azar. The estimation of serum euglobulin may provide a better indication of the severity of the disease in these two conditions than the estimation of total globulin.

The results of repeated determinations of plasma protein values in some of our patients with tuberculosis. kala-azar, cirrhosis and catarrhal jaundice during the course of treatment suggest that Howe's method of fractionation of the proteins of the blood plasma, despite its deficiencies, gives fairly uniform results and may prove to be of great prognostic value in following the course of the disease.

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DETERMINATION OF FLUORINE IN BIOLOGICAL MATERIALS AND ITS APPLICATION IN FLUORINE INTOXICATION STUDIES IN CATTLE IN INDIA.

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THE profound toxic effects of fluorides, even in small quantities, have been clearly recognized in recent times. As fluorine compounds are widespread in nature, attempts have been made in various countries to chart the endemic fluorosis zones. This has led to the working out of a number of methods for analysis of fluorine in biological materials. Of these, the volumetric method of Willard and Winter (1933) has proved to be the most popular. This method is quite simple and with certain modifications (Armstrong, 1933), has been found to be reliable by some workers. Thus, Roholm (1937) found that the method gave results with errors seldom exceeding 10 per cent in samples containing a minimum of 87 μg. of fluorine. During our study of fluorine intoxication cases in cattle in India a search was made for a really dependable micro-method of fluorine estimation, and the Willard and Winter's method was given an exhaustive trial. The results, however, were often found to be inconsistent. duplicate determinations sometimes varying by 50 to 200 per cent. As the method offers several advantages, it was decided to make a more detailed study of it and by scrupulously eliminating the various sources of error to bring it into the line of practical accuracy and general usefulness. The results of our studies are presented in this paper. It may be stated that the Willard and Winter technique modified as a result of these studies admits of fluorine estimation in biological materials in amounts as low as 25 μg. or 30 μg. The error even at this low level scarcely exceeds 5 per cent.

The principle involved in the technique is that when a compound of fluorine is distilled with perchloric acid in the presence of silica under specified conditions, all the fluorine passes into the distillate as hydrofluosilicic acid. The latter is then

fixed in alkali solution, acidified and titrated in alcoholic solution by thorium nitrate employing sodium alizarine sulphonate as an indicator.

EXPERIMENTAL.

Influence of pH and indicator on titre.—While titrating with thorium nitrate solution it was soon realized that the amount of indicator solution added and the acidity of the solution at the time of titration affected the titre considerably. Typical cases are illustrated in Table I :—

TABLE I.

*Influence of the amount of indicator and of pH on titres.**

Amount of NaF solution, c.c.	Indicator, drops.	Extra HCl (1 : 50), drops.	pH.	µg. F. present.	µg. F. found.	Percentage deviation.
1	4	1	3·07	76·0	(1) 64·6	−15·0
					(2) 64·6	−15·0
1	4	3	2·57	76·0	(1) 68·4	−10·0
					(2) 68·4	−10·0
1	4	6	2·34	76·0	(1) 74·5	− 2·0
					(2) 76·0	0·0
1	4	9	2·27	76·0	(1) 79·8	+ 5·0
					(2) 78·3	+ 3·0
1	4	12	2·11	76·0	(1) 87·4	+15·0
					(2) 88·9	+16·1
1	4	15	2·03	76·0	(1) 97·3	+28·0
					(2) 97·3	+28·0
1	5	6	. .	76·0	(1) 69·9	− 8·0
					(2) 71·4	− 6·0
1	3	6	...	76·0	(1) 75·2	− 1·5
					(2) 75·2	− 1·5

* In the above titrations, to 1 c.c. of NaF solution which corresponds theoretically to 1 c.c. of the diluted Th(NO₃)₄ solution, 9 c.c. of distilled water and 10 c.c. of rectified spirit were added and after exact neutralization with HCl solution (as shown by a change of the indicator from pink to colourless or slightly yellow), the extra drops of HCl were added and the titration completed to the first re-appearance of a pink colour.

It will be evident from Table I that both HCl and the indicator solution exerted an influence on the titre. The best and practically theoretical titration values were obtained only when four drops of the indicator solution and six extra drops of the HCl were added. As hydrogen-ion concentration was found to play a definite rôle in the above titrations, the pH values of the various solutions were determined potentiometrically. Using the millilitres of thorium nitrate solution required and the different pH values of the solutions as ordinates and abscissæ respectively, the following Graph has been constructed. The pH at which the best value for fluorine is obtained is 2·34 :—

GRAPH.

Influence of pH value on the titre of thorium nitrate.
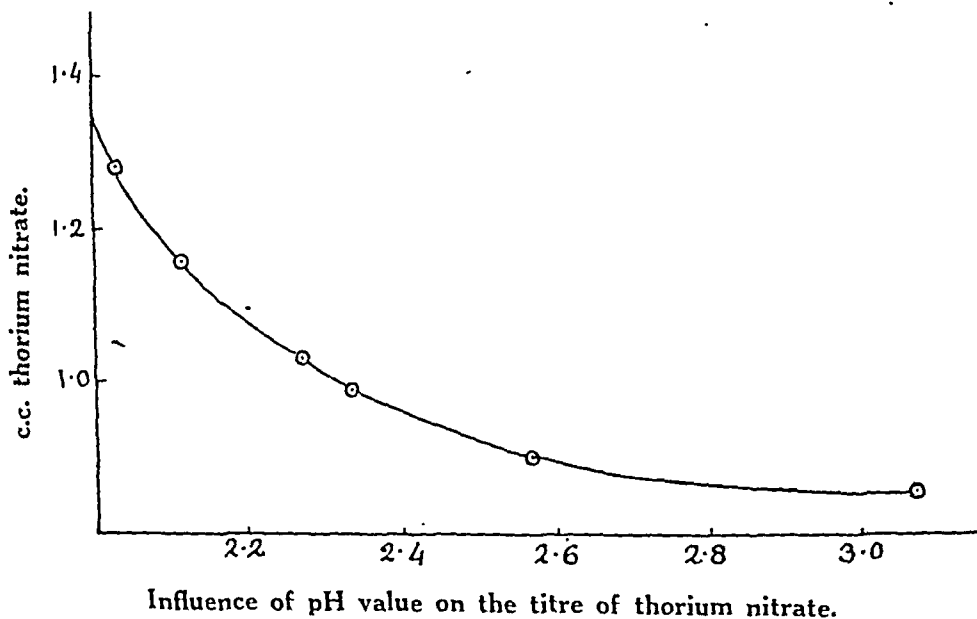
Influence of rectified spirit on titre.—Although the importance of adding rectified spirit in the titrating mixtures has long been recognized. the quantitative aspect did not appear to have been studied with exactitude. For example. it has been stated that equal volume of alcohol should be added but nowhere has it ever been mentioned how the titre was affected when greater or smaller volumes were used. and if at all. An experiment was. therefore. carried out to study the effect of

adding various amounts of rectified spirit on the titre. The results are set out in Table II :—

TABLE II.

Influence of the amount of rectified spirit on titres.[*]

Distillate taken, c.c.	Rectified spirit used, c.c.	Thorium nitrate required, c.c.
10	3	(a) 3·60
10	5	(a) 3·25
		(b) 3·25
10	10	(a) 3·00
		(b) 3·00
10	15	(a) 3·00
		(b) 3·00

* 4 drops of the indicator + 6 extra drops of HCl used.

From Table II it is seen that if less than an equal amount of rectified spirit is used for the titration, the result is apt to be high and that the smaller the amount of alcohol used, the higher was the titre. When an excess of alcohol was used accuracy was not interfered with.

Influence of calcium, phosphate and aluminium ions on titre.—It is known that in the Willard and Winter's method of thorium nitrate titration, the presence of any of the above ions interferes with the result. The quantitative aspect of the ionic interference was considered of interest and experimental evidence was, therefore, gathered to throw light on this. The data are presented in Table III.

TABLE III.

Influence of calcium, phosphate and aluminium ions on direct titration with thorium nitrate.

Amount of NaF solution taken.	µg. F. present.	µg. F. found.	Percentage deviation.
A. *Influence of added calcium ions :—*			
(i) 0·5 c.c. (20 mg. Ca ion added as CaCl₂ in soln.) ...	38·0	26·6	—30·0
(ii) 0·5 c.c. (3 mg. Ca ion added as above)	38·0	31·2	—18·0
(iii) 0·5 c.c. (2 mg. Ca ion added as above)	38·0	33·4	—12·1
(iv) 0·5 c.c. (1 mg. Ca ion added as above)	38·0	35·7	— 6·0
B. *Influence of added phosphate ions :—*			
(i) 0·5 c.c. (1 mg. phosphate ion added as KH_2PO_4 in soln.)	38·0	129·2	+240·0
(ii) 0·5 c.c. (0·1 mg. phosphate ion added as above) ...	38·0	65·4	+ 72·1
C. *Influence of added aluminium ions :—*			
(i) 0·5 c.c. (1 mg. Al ion added as potash alum in soln.)	38·0	Not titrable as the pink colour developed with the addition of the indicator does not seem to disappear even if a large excess of HCl be used.	
D. *Direct titration as such :—*			
(i) 0·5 c.c.	38·0	37·2	— 2·1
(ii) 0·5 c.c.	38·0	38·8	+ 2·1
(iii) 1·0 c.c.	76·0	76·0	0·0

It may be observed from the data in Table III that minute traces of any one of these ions, even with sufficient alcohol in the titrating mixture, introduce a considerable error. But, all these interfering salts can be easily eliminated by perchloric acid distillation, and so this is an essential feature of the method. The perchloric acid distillation, at the outset of this investigation, did not prove to be simple. After numerous trials, details in the technique had to be adjusted and the following precise method was evolved.

THE METHOD OF FLUORINE ESTIMATION.

Apparatus.—The apparatus consists of a distillation set with a Claisen's flask of Pyrex glass having a capacity of 250 c.c., a straight condenser and a receiver

of about 250 c.c. capacity. Rubber-stoppers and rubber-connections were employed everywhere. Through the rubber-stopper attached to the long neck of the flask, a thermometer recording up to 200°C. was inserted down to the bottom of the flask, and through another hole in the stopper a 250-c.c. separating funnel was also fitted. The stem of the funnel was drawn into a capillary which went down to about 2 to 2½ inches from the bottom of the flask. The delivery tube of the distillation flask was bent down vertically and attached to the condenser, with the flask resting on an asbestos board with a hole large enough to expose the bottom of the flask up to a height of ⅛ inch to the even flame from a Fisher burner.

Reagents.—

(1) *Perchloric acid* (Merck's or Baker's).

(2) *N NaOH.*

(3) *Stock thorium nitrate solution :* 1·104 g. Th(NO₃)₄. 4H₂O (Kahlbaum) dissolved in 100 c.c. water. 1 c.c. of stock solution = 1·52 mg. F.

(4) *Dilute thorium nitrate solution :* 5 c.c. of solution No. 3 are made up to 100 c.c. with distilled water, so that 1 c.c. of this diluted solution = 0·076 mg. F.

(5) *Sodium . alizarine sulphonate indicator :* 0·05 per cent of the dye in distilled water.

(6) *Hydrochloric acid :* 1 c.c. pure and concentrated acid made up to 50 c.c.

*Method.—*The ash of the unknown material, or an accurately measured quantity of a standard sodium fluoride solution, was placed in the distillation flask and washed down with a little distilled water. About 5 c.c. of 70 per cent perchloric acid (a correspondingly greater quantity should be added if perchloric acid of a lower strength is used) were added and the apparatus was connected with the condenser. Eighty c.c. of distilled water were then placed in the separating funnel. The burner was now lighted for heating. In order to supply silica to the reacting mixture, as also to prevent bumping, a few glass-beads were put in the flask. Ordinarily, distillation proceeded smoothly, especially with bone, blood, water, etc., but in the case of feeding-stuffs or soils bumping was somewhat violent. This was partially prevented by using broken porcelain pieces instead of glass-beads. When the thermometer recorded a temperature of 140°C., water from the separating funnel was allowed to enter dropwise and at such a rate that the temperature was maintained between 135°C. and 145°C. This was found by trials to be the optimum temperature for the best recovery of the fluorine present. When all the water in the separating funnel had been added, heating was discontinued and the distillate was made just alkaline to litmus with normal caustic soda. The solution was then evaporated to a small bulk in a platinum basin over a sand-bath and finally transferred to a porcelain basin for titration. This method of reducing the bulk has also been adopted by Boruff and Abbott (1933). An equal amount or a small excess of rectified spirit and four drops of the indicator solution were then added, and the solution exactly neutralized with 1 : 50 HCl. Finally, six extra drops of the acid were added and the mixture titrated against the diluted thorium nitrate solution. The titration was performed in bright diffused light and the appearance of a faint, permanent pink colour marked the end-point.

Recovery.—In Table IV is detailed the result of recovery determinations which fully supports the accuracy of the method :—

TABLE IV.

Recovery of fluorine on perchloric acid distillation.

Substance taken.	μg. F. present.	μg. F. found.	Percentage deviation.
Direct titration :—			
(i) 0·5 c.c. NaF solution	38·0	37·2	−2·1
(ii) 1·0 c.c. NaF solution	76·0	74·4	−2·1
(iii) 2·0 c.c. NaF solution	152·0	155·8	+2·5
After distillation :—			
(iv) 0·5 c.c. NaF solution (5·0 c.c. 70 per cent perchloric acid used).	38·0	38·0	0·0
(v) 0·5 c.c. NaF solution (20·0 c.c. 20 per cent acid used).	38·0	36·5	−3·9
(vi) 2·0 c.c. NaF solution (20·0 c.c. 20 per cent acid used).	152·0	158·1	+4·0
(vii) 10 mg. CaF_2	4,870	(a) 4,940·0	+1·4
		(b) 5,035·0	+3·4
(viii) 20 mg. CaF_2	9,740	(a) 10,070·0	+3·4
		(b) 10,200·0	+4·7
(ix) (a) 0·3 g. tooth ash	...	440·8	...
(b) 0·3 g. tooth ash + 20 mg. CaF_2 ...	10,180·8	10,533·6	−2·5
(x) (a) 0·3 g. tooth ash (different sample) ...	...	684·0	...
(b) 0·3 g. tooth ash as above + 20 mg. CaF_2 ...	10,819·8	10,928·0	+5·0

ATTEMPT AT REPLACEMENT OF PERCHLORIC ACID BY SULPHURIC ACID.

The high cost of perchloric acid and its non-availability during war years offered considerable difficulties in the course of this investigation. Following the lead of Hoffmann and Lundell (1938 ; quoted by Low and Pryde, 1939), who previously used sulphuric acid instead of perchloric acid for a similar purpose,

an attempt in this direction was made.   This, however, was unsuccessful, as Table V will show :—

<div align="center">TABLE V.</div>

Effect of replacing perchloric acid by sulphuric acid in the distillation process.

Substance.	THORIUM NITRATE SOLUTION REQUIRED ON		
	Perchloric acid distillation, c.c.	Sulphuric acid distillation, c.c.	Percentage deviation (on the basis of perchloric acid distillation).
0·15 g. bone ash	1·46	1·57	+ 8·0
0·15 g. as above + 38 µg. F. as NaF	1·96	1·75	−10·0
0·30 g. ash as above	2·92	2·20	−44·0
0·5 c.c. NaF solution = 38 µg. F.	0·50	0·72	+44·0
0·07 g. bone ash (different sample)	1·42	1·40	− 1·0
0·07 g. ash + 38 µg. F. as NaF	1·95	1·70	−10·0
0·15 g. ash as above	3·05	2·10	−30·0
0·15 g. as above + 38 µg. F. as NaF	3·55	2·15	−40·0

PREPARATION OF MATERIAL.

For perchloric acid distillation, it is essential that the material to be distilled is in the form of ash, otherwise, the procedure is beset with difficulties and danger. The presence of organic matter leads to the formation of chlorine fumes and explosion may occur. The procedures adopted for the preparation of products that can safely be distilled vary with different biological materials and are described below. In this connection the book by Roholm (*loc. cit.*) has been freely consulted.

(a) *Bones and teeth.*—Bones and teeth from recently-killed animals or from field cases preserved in spirit were mechanically cleaned by removal of the adhering tissues and then left in water containing 0·5 per cent trypsin or papain for 4 to 6 hours at 37°C. or 50°C., as the case may be, for incubation (Subrahmanyan, Duckworth and Godden, 1939). After all soft tissues had been dissolved, the specimen was washed with a little water, refluxed with alcohol for about six hours and finally soxhleted with sulphuric ether for another six hours. After this, the de-fatted material was ground in an iron mortar and left in a platinum basin at 105°C. to dry. The dried material was then carefully incinerated and ashed in an electric furnace at about 500°C. The ash was then ready for distillation.

(b) *Blood.*—A known volume, say 40 c.c., was mixed with an amount of analytical quality calcium oxide to correspond to 2 per cent strength by volume as a fluorine fixative and finally stirred with an equal volume of absolute alcohol. This was then dried on a water-bath, incinerated and ashed; the furnace temperature was not allowed to exceed 500°C.

(c) *Flesh.*—A quantity of finely minced flesh was taken and treated in the same way as bones and teeth from the alcohol refluxing stage. The fat-free dry material was mixed with 2 per cent calcium acetate as a fixative and the whole was moistened with a little water subsequently. The usual drying and ashing were done.

(d) *Water.*—A known volume, say 250 c.c., was shaken with an amount of calcium hydroxide (*pro analysi* quality) to correspond to 2 per cent strength by volume. This was then evaporated to dryness by transferring gradually and quantitatively the same to a platinum basin heated on a sand-bath. Finally, the dried residue was incinerated, ashed and distilled in the usual way.

For the estimation of fluorine in water, at first the simpler method of alkalinizing and evaporating to dryness, and finally distilling with perchloric acid was adopted. It was found, however, that chlorine fumes were evolved and the distillation was accompanied by violent bumping. Further, it was found that normal thorium nitrate titration was considerably interfered with and the indicator was bleached. At this stage, we came across a paper by Lockwood (1937), in which the use of sodium nitrate and aeration was suggested as a means of getting rid of chlorine, thus avoiding all interference with the normal titration procedures. Acting on this suggestion, the difficulties in titration could be avoided, but the chlorine fumes and bumping during the course of distillation still remained as sources of great inconvenience. Ashing, on the contrary, of the dried residue removed this inconvenience and hence this was adopted for the water distillation. In this connection, it may be mentioned that Lockwood (*loc. cit.*) attributed the formation of chlorine fumes during the distillation to the presence of manganese in the original water. As ashed material did not give rise to these fumes, it may reasonably be concluded that the organic matter present in the water and not manganese was the factor responsible.

(e) *Fodder and soil.*—A known amount of the dried substance was taken and its moisture determined. The finely powdered material was then thoroughly mixed with 2 per cent calcium acetate and a little water. This was next slowly incinerated and ashed. It is better here to work with the minimum quantity of the ash, otherwise, there is a lot of bumping. Addition of broken porcelain pieces to the distilling fluid instead of glass-beads was found helpful in preventing or at least in minimizing the bumping.

(f) *Faeces and urine.*—For faeces, calcium acetate was used as a fixative, while for urine the fixative used was calcium hydroxide. The rest of the procedure was the same as in the case of feeding-stuffs and water, respectively.

SURVEY OF THE FLUORINE CONTENTS IN PLANTS AND ANIMAL TISSUES.

The next stage in the investigation consisted of a survey of the distribution of fluorine in nature, especially in normal plant and animal tissues. Very little

systematic work has been done on these aspects in India.   On the other hand, as it is generally supposed that in fluorosis fluorine accumulates in the tissues of the diseased animals, a study of the fluorine content of the tissues of normal animals was considered essential for purposes of comparison with those of animals suffering from fluorine toxicosis.   The possibility that such a study might be useful in the post-mortem diagnosis of cases of fluorosis was of interest.   Similarly, an investigation into the fluorine content of water, soil and fodder was also indicated in order to find out the relative importance of the various substances as sources of intoxication in the causation of the disease.

In what follows, an attempt will be made to describe the results of work done in these laboratories and its usefulness in fluorine intoxication studies in cattle in India.

Table VI gives the amount of fluorine present in the bones and tissues of normal cattle.   Parallel figures for fluorotic animals, both from our own experiments and from the field, are also given.   For convenience, average figures and range only have been indicated.   It will be seen that the figures from fluorotic animals are invariably higher than the maximum fluorine value of normal tissues.

TABLE VI.

Fluorine concentration in bone, blood and flesh of normal and fluorotic animals.

Tissue.	Number of samples examined.	Normal.	Fluorotic.
Blood :— F mg./100 c.c. whole blood (average) ...	56 normal and 5 fluorotic	0·13 (0·08–0·23)	0·82 (0·37–1·88)
Bone :— F mg./100 g. dry fat-free material (average)	(a) Pelvis 10 normal and 4 fluorotic	123·9 (27·0–427·4)	787·0 (459·4–1,115·5)
	(b) Molar 7 normal and 7 fluorotic	42·3 (15·2–94·3)	180·2 (93·9–317·2)
Flesh :— F mg./pound of fresh beef (average) ...	3 normal and 3 fluorotic	0·33 (0·32–0·34)	3·45 (1·14–7·75)

The estimation of fluorine in the tissues, especially in bones, is, therefore, a guide to the incidence of fluorosis and in locating the enzoötic fluorosis zones.

In Table VII the concentration of fluorine in water soil and feeding-stuffs from both enzoötic and non-enzoötic fluorosis zones is given :—

TABLE VII.

Fluorine concentration in water, soil and feeding-stuffs from affected and non-affected localities.

Substance examined.	Total Number of samples examined.	Affected.	Non-affected.	Suspected.*
Water :— F mg./litre	24	1·50 (1·10–2·01)	0·44 (0·10–0·72)	1·38 (0·84–2·60)
Soil :— F mg./kg. of dried matter ...	3	103·0 (88·0–118·0)	78·0 ...	... ...
Kharimatti (chalk) :— F mg./kg. of dried matter ...	9	150·0 (142·0–158·0)	... ...	128·0 (102·0–156·0)
Feeding-stuffs :— F mg./kg. of dried stuffs—				
1 Wheat bhoosa	1	...	3·50	...
2 Wheat bran	1	...	7·90	...
3 Barley	2	...	3·69	...
4 Gram	1	4·10	4·32	...
5 Ground-nut cake ...	1	0·98	1·02	...
6 Rice straw	1	3·33	...	...
Rice-straw alkali treated ...	1	2·93	...	...
7 Ground-nut hay... ...	1	3·33	...	...
8 Jowar straw	1	3·13	...	...
9 Jowar husk	1	3·00	...	...
10 Veregi straw	1	2·27	...	...

* ' Suspected ' includes areas where the incidence of fluorosis has lately been detected as a result of examination of samples of bone, etc., in these laboratories.

It may be seen from the data in Table VII that in the enzoötic areas the fluorine concentration in water and soil is relatively higher, but this is not noticeable in the feeding-stuffs.

A few data have also been collected with respect to the normal physiological excretion of fluorine in cattle maintained in areas where the fluorine concentration in water is fairly low as at Izatnagar. The hill-bulls in this experiment were fed on a ration consisting of wheat bhoosa as roughage, and ground-nut cake and barley as concentrates. The daily average excretion of fluorine through urine and fæces were respectively 3·73 mg. (3·06 mg. to 4·85 mg.) and 5·33 mg. (5·08 mg. to 5·72 mg.).

SUMMARY.

1. An exhaustive study of the Willard and Winter's technique of fluorine estimation has been made and improvements suggested.

2. The improved technique is capable of estimating fluorine in amounts as low as 25 or 30 microgram with an error not exceeding ± 5 per cent.

3. Examination of a large number of body tissues, viz., bone, blood and flesh, shows a considerable accumulation of fluorine in the tissues of affected animals, and it is claimed that the examination, particularly of the concentration of fluorine in the pelvic bone and molars, can be usefully employed in detecting fluorosis cases and localities.

4. High fluorine concentrations are detectable in the water and soil samples but not in the feeding-stuffs of enzoötic areas.

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